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APPLICATION NUMBER: *60/552,318*
FILING DATE: *March 10, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/07704*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

Docket Number	UCSD-08812	Type a plus sign (+) inside this box →	+

INVENTOR(s) / APPLICANT(s)

Last Name	First Name	Middle Initial	Residence (City and Either State or Foreign Country)
Beattie	**Gillian**		**San Diego, California**
Hayek	**Alberto**		**La Jolla, California**
Lopez	**Ana**		**San Diego, California**

TITLE OF THE INVENTION (280 Characters Max.)

ACTIVIN A MAINTAINS PLURIPOTENTIALITY OF HUMAN EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER LAYERS

CORRESPONDENCE ADDRESS

MEDLEN & CARROLL, LLP
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United States of America

ENCLOSED APPLICATION PARTS *(Check All That Apply)*

X	Specification	Number of Pages	52	X	Assignment (unexecuted)
X	Drawing(s)	*Number of Sheets*	20	X	Power of Attorney (unexecuted)
				X	Declaration (unexecuted)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

___	Charge Account No. **08-1290** in the amount of **$80.00. An originally executed duplicate of this transmittal is enclosed for this purpose.**	FILING FEE AMOUNT ($)	**$80.00**
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This invention was made by an agency of the United States Government under a contract with an agency of the United States Government.

X No.

___ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Date: March 10, 2004

Maha Hamdan

Maha A. Hamdan
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___ Additional inventors are being named on separately numbered sheets attached hereto.

Express Mail Label: **EV 329 476 576 US**

PATENT
Attorney Docket No.: **UCSD-08812**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Gillian Beattie, Alberto Hayek and Ana Lopezi

For: **ACTIVIN A MAINTAINS PLURIPOTENTIALITY OF HUMAN EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER LAYERS**

MS Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on **March 10, 2004**, in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. § 1.10, Mailing Label Number **EV 329 476 576 US** addressed to: **MS Provisional Patent Application**, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Karen B. Webber

Karen B. Webber

TRANSMITTAL COVER SHEET FOR FILING PROVISIONAL APPLICATION (37 C.F.R. § 1.51(2)(i))

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

1. The following comprises the information required by 37 C.F.R. § 1.51(a)(2)(i)(A):

2. The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(a)(2)(i)(B)):

   Gillian Beattie
   Alberto Hayek
   Ana Lopez

3. Address(es) of the inventor(s), as numbered above (37 C.F.R. § 1.51(a)(2)(i)(C)):

   3545 Caminito El Rincon #237, San Diego 92130
   8818 Nottingham Place, La Jolla, 92037
   11115 Adriatic Place, San Diego 92126

4. The title of the invention is (37 C.F.R. § 1.51(a)(2)(i)(D)):

   ACTIVIN A MAINTAINS PLURIPOTENTIALITY OF HUMAN EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER LAYERS

5. The name, registration, and telephone number of the attorney (*if applicable*) is (37 C.F.R. § 1.51(a)(2)(i)(E)):

Maha A. Hamdan
Reg. No.: 43,655
Tel.: (415) 904-6500

(complete the following, if applicable)

_X_ A Power of Attorney (unexecuted) accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. § 1.51(a)(2)(i)(F)):

Docket No.: **UCSD-08812**

7. The correspondence address for this application is (37 C.F.R. § 1.51(a)(2)(i)(G)):

MEDLEN & CARROLL, LLP
101 Howard Street, Suite 305
San Francisco, California 94105

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(a)(2)(i)(H)):

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

_X_ No.

___ Yes.

The name of the U.S. Government agency and the Government contract number are: __.

9. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. § 1.51(a)(2)(ii)-(iii):

Specification: No. of pages **52**

Drawings: No. of sheets **20**

B. Additional documents:

___ Claims: No. of claims __

_X_ Power of Attorney (unexecuted)

_X_ Assignment (unexecuted)

_X_ Declaration (unexecuted)

Express Mail Label: **EV 329 476 576 US**

PATENT
Attorney Docket No.: **UCSD-08812**

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is $160.00, for other than a small entity, and $80.00, for a small entity.

__X__ Applicant is a small entity.

11. Small Entity Statement

__X__ This is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27.

12. Fee payment being made at this time

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Date: March 10, 2004

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ACTIVIN A MAINTAINS PLURIPOTENTIALITY OF HUMAN EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER LAYERS

FIELD OF THE INVENTION

The present invention relates to maintenance of the undifferentiated state and/or pluripotency in embryonic stem cells, and in particular, maintaining undifferentiated human embryonic stem cell lines.

BACKGROUND

New treatments for human disease such as gene therapy and for the replacement of defective or lost body tissues and organs are based upon the potential use of stem cells. However, maintaining these undifferentiated pluripotent stem cells in culture, a necessity for this type of research and subsequently for treatment, is notoriously difficult. When placed in culture, undifferentiated cells spontaneously begin rapid differentiation and typically slow or stop dividing. Thus few stem cells are actually available for research and treatments.

Maintenance of the undifferentiated state and pluripotency in mouse embryonic stem (ES) cells requires the presence of mouse fibroblast feeder layers (mEFs) or activation of STAT3 with leukemia inhibitory factor (LIF). Human embryonic stem cell lines (hES) have only recently become available for research, and the intracellular pathways for self renewal and differentiation are, at this time, largely unknown. Recently it was discovered that activation of STAT3 is not sufficient to block differentiation of human ES cell lines when grown on mEFs or when treated with conditioned media from mEFs.

Thus, there is a need to find ways to maintain undifferentiated stem cells in culture and to increase their proliferation.

SUMMARY OF THE INVENTION

The present invention relates to maintenance of the undifferentiated state and/or pluripotency in embryonic stem cells, and in particular, maintaining undifferentiated human embryonic stem cell lines, using culture medium enriched with Activin A and keratinocyte growth factor, without using fibroblast feeder layers or leukemia inhibitory factor. In one embodiment, the present invention relates to compositions for the maintenance of the undifferentiated state with Activin A and for increasing proliferation with keratinocyte growth factor.

In one embodiment, the invention provides a composition comprising activin A (ACTa) and keratinocyte growth factor (KGF), wherein the concentration of ACTa in said composition maintains embryonic stem cells in an undifferentiated state, and the concentration of KGF in said composition maintains proliferation of embryonic stem cells.

In one embodiment, the invention provides a composition comprising a first polypeptide having at least 90% identity to SEQ ID NO:1 and a second polypeptide having at least 90% homology to SEQ ID NO:17, wherein the concentration of said first polypeptide in said composition maintains embryonic stem cells in an undifferentiated state, and the concentration of said second polypeptide in said composition maintains proliferation of embryonic stem cells. Accordingly, in other embodiments, the first polypeptide is at least 90%, 95%, 96%, 97%, 98%, 99% (or more) identical to any of SEQ ID NOs: 1, 3-16. Accordingly in other embodiments, the second polypeptide is at least 90%, 95%, 96%, 97%, 98%, 99% (or more) identical to any of SEQ ID NOs: 17-24.

In one embodiment, the invention provides a method for maintaining and/or proliferating mammalian embryonic stem (ES) cells, comprising: a) providing; i) ES cells of interest; and ii) medium comprising one or more of activin A and/or keratinocyte growth factor; and b) contacting said ES cells of interest with said medium to produce contacted ES cells. Such methods provide a means to maintain undifferentiated stem cells, a means to promote cell proliferation through cell division and a means to maintain cells in an

undifferentiated state while promoting proliferation in order to increase numbers of undifferentiated cells. In another embodiment, the invention provides a method wherein said contacting is in the absence of mouse fibroblast feeder cells. In another embodiment, the invention provides a method wherein said contacting is in the absence of conditioned medium from mouse fibroblast feeder cells. In another embodiment, the invention provides a method wherein said contacting is in the absence of leukemia inhibitory factor. It is not intended that the present invention be limited to the manner in which the stem cells are maintained and grown. In certain embodiments, fresh biopsied materials are employed to provide stem cells. In other embodiments, cultured stem cells are employed. With regard to the latter, it is not intended that the present invention be limited by the particular culturing method of culturing materials. In one embodiment, the stem cells used in the method are cultured in serum-free culture medium. In one embodiment, the stem cells used in the method are cultured in DSR medium with supplementation (as described in Example 1). In another embodiment, the invention provides a method wherein said activin A is recombinant. In another embodiment, the invention provides a method wherein said recombinant activin A comprises a polypeptide at least 90% identical to SEQ ID NO:1. Accordingly in other embodiments, activin A polypeptides are at least 90%, 95%, 96%, 97%, 98%, 99% (or more) identical to any of SEQ ID NOs: 1, 3-16. In another embodiment, the invention provides a method wherein said activin A is human. In another embodiment, the invention provides a method wherein said keratinocyte growth factor is human. In another embodiment, the invention provides a method wherein said activin A is from one or more of SEQ ID NOs: 1, 3-16. In another embodiment, the invention provides a method wherein said activin A has a concentration of 2nM in said medium. In some embodiments, said composition comprises 0.01 nM, 0.1 nM, 0.5 nM, 1 nM, 2 nM, 3 nM, 4 nM, 8 nM, 16 nM, 32 nM, 64 nM (or any amount between 0.01 nM and 64 nM) of activin A. In another embodiment, the invention provides a method wherein said keratinocyte growth factor is recombinant.In another embodiment, the invention provides a method wherein said recombinant keratinocyte growth factor comprises a

polypeptide at least 90% identical to SEQ ID NO:17. In another embodiment, the invention provides a method wherein said keratinocyte growth factor is from one or more of SEQ ID NOs:11-14. Accordingly in other embodiments, the second polypeptide is at least 90%, 95%, 96%, 97%, 98%, 99% (or more) identical to any of SEQ ID NOs: 17-24. In another embodiment, the invention provides a method wherein said keratinocyte growth factor has a concentration of 50 ng/ml in said medium. In some embodiments, said composition comprises 0.50 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 50 ng/ml, 75 ng/ml, 100 ng/ml, 200 ng/ml, 500 ng/ml, 1 mg/ml (or any amount between 0.50 ng/ml, and 1 mg/ml) of keratinocyte growth factor. In another embodiment, the invention provides a method wherein said ES cells of interest are mammalian. In another embodiment, the invention provides a method wherein said ES cells of interest are human. It is not meant to limit the types of stem cells. A variety of stem cell types can be cultured by the compositions and methods of the present invention, including but not limited to stem cells selected from the group consisting of embryonic, fetal and adult stem cells. It is not intended that the present invention be limited to the sources of stem cells. In one embodiment, stem cells are derived from embryonic tissue. In one embodiment, stem cells are derived from fetal tissue. In one embodiment, stem cells are derived from adult tissue. In one embodiment, stem cells are derived from blood. It is not meant to limit the species of stem cell cultured with the compositions and methods of the present invention. A variety of species may be used as sources of stem cells, including but not limited to human, great apes, monkeys, cows, horses, sheep, pigs, goats, dogs, cats, guinea pigs, rats, mice, goldfish, xenopus, zebrafish, *etc.*. It is not meant to limit the differentiation state of the contacted cells. In another embodiment, the invention provides a method wherein said ES cells of interest are undifferentiated. In another embodiment, the invention provides a method wherein said ES cells of interest are differentiated. In another embodiment, the invention provides a method wherein said contacted ES cells are undifferentiated. In another embodiment, the invention provides a method wherein said contacted ES cells are pluripotent. In another embodiment, the invention provides a method wherein said contacted cells are

pluripotent *in vivo*. In another embodiment, the invention provides a method wherein said contacted ES cells are pluripotent *in vitro*. In another embodiment, the invention provides a method wherein said contacted ES cells have the same karyotype as said ES cells of interest.

In one embodiment the invention provides a method for maintaining and/or proliferating mammalian embryonic stem (ES) cells: a) providing; i) ES cells of interest; and ii) medium comprising one or more of a first polypeptide having at least 90% identity to SEQ ID NO:1 and/or a second polypeptide having at least 90% homology to SEQ ID NO:17; and; b) contacting said ES cells of interest *in vitro* with said medium to produce contacted ES cells. In another embodiment, the invention provides a method wherein the concentration of said first polypeptide in said medium maintains said contacted ES cells in an undifferentiated state. In another embodiment, the invention provides a method wherein the concentration of said second polypeptide in said medium maintains proliferation of said contacted ES cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a shows exemplary embodiments demonstrating morphology and differentiation state of HSF6 cells observed by phase contrast microscopy (upper layer), and immunohistochemistry (lower layer). MEF = mouse embryonic fibroblasts, LAM=laminin, ACT = Activin A, KGF= keratinocyte growth factor, FOL= Follistatin. Magnification bar = 100 μM.

Figure 1b shows exemplary embodiments demonstrating semi-quantitative RT-PCR of hES cells for oct-4, nanog and telomerase under a variety of culture conditions on mEFs (lane 1 and 6), or on laminin (lane 2-5).

Figure 1c shows exemplary embodiments demonstrating expression of activin in mEFs using RT-PCR and western blots. +/-: reverse transcriptase.

Figure 2a shows exemplary embodiments demonstrating teratoma formation in nude mice.

Figure 2b shows exemplary embodiments demonstrating RT-PCR analysis of 17 day old embryoid bodies from hES previously cultured on laminin with activin and KGF for 8 passages. Neuro D =ectoderm, T gene= mesoderm, å-FP = endoderm.

Figure 3 shows SEQ ID NO:01: Inhibin beta A subunit (Activin A) *Homo sapiens* (PeproTech and GenBank X04447) amino acid sequence.

Figure 4 shows SEQ ID NO:02: Inhibin beta A chain (Activin beta-A chain) *Homo sapiens* (GenBank X04447) macrophage cell line U937 (ATCC CRL 1539) nucleic acid sequence.

Figure 5 shows SEQ ID NO:3-6 comprising amino acid sequences of human activin A.

Figure 6 shows SEQ ID NO:7-16 comprising amino acid sequences of animal activin A.

Figure 7 shows SEQ ID NO:17: keratinocyte growth factor (PeproTech) - Homo sapiens (Human) amino acid sequence amino acid sequence; SEQ ID NO:18: Keratinocyte growth factor (Swiss-Prot P21781) (GenBank M60828; S81661) - Homo sapiens (Human) amino acid sequence; SEQ ID NOs:19-24 keratinocyte growth factor amino acid sequences from animals.

Figure 8 shows SEQ ID NO:25-31 keratinocyte growth factor nucleic acid sequences.

Figure 9 shows SEQ ID NO:32-35 activin A nucleic acid sequences.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein including within this specification and the appended claims, the forms "a," "an" and "the" includes both singular and plural references unless the content clearly dictates otherwise.

As used herein, the term "or" when used in the expression "A or B," and where A and B refer to a composition, disease, product, *etc.*, means one, or the other, or both.

As used herein, the term "comprising" when placed before the recitation of steps in a method means that the method encompasses one or more steps that are additional to those expressly recited, and that the additional one or more steps may be performed before, between, and/or after the recited steps. For example, a method comprising steps a, b, and c encompasses a method of steps a, b, x, and c, a method of steps a, b, c, and x, as well as a method of steps x, a, b, and c. Furthermore, the term "comprising" when placed before the recitation of steps in a method does not (although it may) require sequential performance of the listed steps, unless the content clearly dictates otherwise. For example, a method comprising steps a, b, and c encompasses, for example, a method of performing steps in the order of steps a, c, and b, the order of steps c, b, and a, and the order of steps c, a, and b, *etc.*

The terms "antibody" and "immunoglobulin" are interchangeably used to refer to a glycoprotein or a portion thereof (including single chain antibodies), which is evoked in an animal by an immunogen and which demonstrates specificity to the immunogen, or, more specifically, to one or more epitopes contained in the immunogen. The term "antibody" includes polyclonal antibodies, monoclonal antibodies, blocking antibodies, neutralizing antibodies, inhibiting antibodies, stimulating antibodies, naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof, including, for example, Fab, $F(ab')_2$, Fab fragments, Fd fragments, and Ev fragments of an antibody, as well as a Fab expression library. It is intended that the term "antibody" encompass any immunoglobulin (*e.g.*, IgG, IgM, IgA, IgE, IgD, *etc.*) obtained from any source (*e.g.*, humans, rodents, non-human primates, caprines, bovines, equines, ovines, *etc.*). The term "polyclonal antibody" refers to an immunoglobulin produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an immunoglobulin produced from a single clone of plasma cells. Monoclonal and polyclonal antibodies may or may not be purified. For example, polyclonal antibodies contained in crude antiserum may be used in this unpurified state.

Naturally occurring antibodies may be generated in any species including murine, rat, rabbit, hamster, human, and simian species using methods known in the art. Non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as previously described [Huse *et al.*, Science 246:1275-1281 (1989)]. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward *et al.*, Nature 341:544-546 (1989); Hilyard *et al.*, Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

Those skilled in the art know how to make polyclonal and monoclonal antibodies which are specific to a desirable polypeptide. For the production of monoclonal and polyclonal antibodies, various host animals can be immunized by injection with the peptide corresponding to any molecule of interest in the present invention, including but not limited to rabbits, mice, rats, sheep, goats, chickens, *etc.* In one preferred embodiment, the peptide is conjugated to an immunogenic carrier (*e.g.*, diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH)). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward molecules of interest in the present invention, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (*See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY). These include but are not limited to the hybridoma technique originally developed by Köhler and

Milstein (Köhler and Milstein, Nature 256:495-497 [1975]), as well as the trioma technique, the human B-cell hybridoma technique (*See e.g.*, Kozbor *et al.* Immunol. Today 4:72 [1983]), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al., in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 [1985]). In some particularly preferred embodiments of the present invention, the present invention provides monoclonal antibodies of the IgG class.

In additional embodiments of the invention, monoclonal antibodies can be produced in germ-free animals utilizing technology such as that described in PCT/US90/02545. In addition, human antibodies may be used and can be obtained by using human hybridomas (Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A.80:2026-2030 [1983]) or by transforming human B cells with EBV virus *in vitro* (Cole *et al., in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96 [1985]).

Furthermore, techniques described for the production of single chain antibodies (*See e.g.*, U.S. Patent 4,946,778; herein incorporated by reference) can be adapted to produce single chain antibodies that specifically recognize a molecule of interest (*e.g.*, at least a portion of an AUBP or mammalian exosome, as described herein). An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.,* Science 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a particular protein or epitope of interest (*e.g.*, at least a portion of an AUBP or mammalian exosome).

The invention also contemplates humanized antibodies. Humanized antibodies may be generated using methods known in the art, including those described in U.S. Patent Numbers 5,545,806; 5,569,825 and 5,625,126, the entire contents of which are incorporated by reference. Such methods include, for example, generation of transgenic non-human animals which contain human immunoglobulin chain genes and which are capable of expressing these genes to produce a repertoire of antibodies of various isotypes encoded by the human immunoglobulin genes.

According to the invention, techniques described for the production of single chain antibodies (U.S. 4,946,778; herein incorporated by reference) can be adapted to produce specific single chain antibodies as desired. An additional embodiment of the invention utilizes the techniques known in the art for the construction of Fab expression libraries (Huse *et al.*, Science, 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments that contain the idiotype (antigen binding region) of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment that can be produced by pepsin digestion of an antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of an F(ab')2 fragment, and the Fab fragments that can be generated by treating an antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA [enzyme-linked immunosorbent assay], "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays [*e.g.*, using colloidal gold, enzyme or radioisotope labels], Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*

The terms "antisense DNA sequence" and "antisense sequence" as used herein interchangeably refer to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Sense mRNA generally is ultimately translated into a polypeptide. Thus, an "antisense DNA sequence" is a sequence which has the same sequence as the non-coding strand in a DNA duplex, and which encodes an "antisense RNA" (*i.e.*, a ribonucleotide

sequence whose sequence is complementary to a "sense mRNA" sequence). The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand. Antisense RNA may be produced by any method, including synthesis by splicing an antisense DNA sequence to a promoter which permits the synthesis of antisense RNA. The transcribed antisense RNA strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation, or promote its degradation.

Antisense oligonucleotide sequences may be synthesized using any of a number of methods known in the art (such as solid support and commercially available DNA synthesizers, standard phosphoramidate chemistry techniques, and commercially available services, *e.g.*, Genta, Inc.).

As used herein, the term "RNAi" and "RNA interference" refers to the ability of double stranded RNA to suppress the expression of a gene corresponding to its own sequence. Ribozyme sequences have been successfully used to inhibit the expression of several genes including the gene encoding VCAM1, which is one of the integrin $\alpha 4\beta 1$ ligands [U.S. Patent No. 6,252,043, incorporated in its entirety by reference]. The term "ribozyme" refers to an RNA sequence that hybridizes to a complementary sequence in a substrate RNA and cleaves the substrate RNA in a sequence specific manner at a substrate cleavage site. Typically, a ribozyme contains a "catalytic region" flanked by two "binding regions." The ribozyme binding regions hybridize to the substrate RNA, while the catalytic region cleaves the substrate RNA at a "substrate cleavage site" to yield a "cleaved RNA product."

As used herein, the term "contacting" cells with an agent or antibody refers to placing the agent or a antibody in a location that will allow it to touch the cell in order to produce "contacted" cells. The contacting may be accomplished using any suitable method. For example, in one embodiment, contacting is by adding the agent or a antibody to a tube of cells. Contacting may also be accomplished by adding the agent to a culture of the cells. It

is not meant to limit how the agent or antibody contacts the cells. In one embodiment, contacting may be accomplished by administration of agent or antibody to an animal *in vivo*.

As used herein, the terms "antigen," "immunogen," "antigenic," "immunogenic," "antigenically active," and "immunologically active" refer to any substance that is capable of inducing a specific humoral or cell-mediated immune response. An immunogen generally contains at least one epitope. Immunogens are exemplified by, but not restricted to molecules which contain a peptide, polysaccharide, nucleic acid sequence, and/or lipid. Complexes of peptides with lipids, polysaccharides, or with nucleic acid sequences are also contemplated, including (without limitation) glycopeptide, lipopeptide, glycolipid, *etc.* These complexes are particularly useful immunogens where smaller molecules with few epitopes do not stimulate a satisfactory immune response by themselves.

As used herein, the term "cell" refers to a single cell as well as to a population of (*i.e.,* more than one) cells. The population may be a pure population comprising one cell type. alternatively, the population may comprise more than one cell type. In the present invention, there is no limit on the number of cell types that a cell population may comprise.

As used herein, the term "cell culture" refers to any *in vitro* culture of cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, finite cell lines (*e.g.*, non-transformed cells), and any other cell population maintained *in vitro*, including oocytes and embryos.

As used herein, the term "mixed cell culture," refers to a mixture of two or more types of cells. In some embodiments, the cells are cell lines that are not genetically engineered, while in other embodiments the cells are genetically engineered cell lines. In some embodiments the cells contain genetically engineered molecules. The present invention encompasses any combination of cell types suitable for the detection, identification, and/or quantitation of apoptosis in samples, including mixed cell cultures in which all of the cell types used are not genetically engineered, mixtures in which one or more of the cell types are

genetically engineered and the remaining cell types are not genetically engineered, and mixtures in which all of the cell types are genetically engineered.

As used herein, the term "primary cell" is a cell which is directly obtained from a tissue (*e.g.* blood) or organ of an animal in the absence of culture. Typically, though not necessarily, a primary cell is capable of undergoing ten or fewer passages *in vitro* before senescence and/or cessation of proliferation. In contrast, a "cultured cell" is a cell which has been maintained and/or propagated *in vitro* for ten or more passages.

As used herein, the term "cultured cells" refer to cells which are capable of a greater number of passages *in vitro* before cessation of proliferation and/or senescence when compared to primary cells from the same source. Cultured cells include "cell lines" and "primary cultured cells."

As used herein, the term "cell line," refers to cells that are cultured *in vitro*, including primary cell lines, finite cell lines, continuous cell lines, and transformed cell lines. but does not require, that the cells be capable of an infinite number of passages in culture. Cell lines may be generated spontaneously or by transformation.

As used herein, the terms "primary cell culture," and "primary culture," refer to cell cultures that have been directly obtained from cells *in vivo*, such as from animal or insect tissue. These cultures may be derived from adults as well as fetal tissue.

As used herein, the terms "monolayer," "monolayer culture," and "monolayer cell culture," refer to cells that have adhered to a substrate and grow as a layer that is one cell in thickness. Monolayers may be grown in any format, including but not limited to flasks, tubes, coverslips (*e.g.*, shell vials), roller bottles, *etc.* Cells may also be grown attached to microcarriers, including but not limited to beads.

As used herein, the term "suspension" and "suspension culture" refers to cells that survive and proliferate without being attached to a substrate. Suspension cultures are typically produced using hematopoietic cells, transformed cell lines, and cells from malignant tumors.

As used herein, the terms "culture media," and "cell culture media," refers to media that are suitable to support the growth of cells *in vitro* (*i.e.*, cell cultures). It is not intended that the term be limited to any particular culture medium. For example, it is intended that the definition encompass outgrowth as well as maintenance media. Indeed, it is intended that the term encompass any culture medium suitable for the growth of the cell cultures of interest.

As used herein the term, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *in vitro* environments exemplified, but are not limited to, test tubes and cell cultures. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reactions that occur within a natural environment.

As used herein, the term "proliferation" refers to an increase in cell number.

As used herein, the term "differentiation" refers to the maturation process cells undergo whereby they develop distinctive characteristics, and/or preform specific functions, and/or are less likely to divide.

As used herein, the terms "isolated," "to isolate," "isolation," "purified," "to purify," "purification," and grammatical equivalents thereof as used herein, refer to the reduction in the amount of at least one contaminant (such as protein and/or nucleic acid sequence) from a sample. Thus purification results in an "enrichment," *i.e.*, an increase in the amount of a desirable protein and/or nucleic acid sequence in the sample.

As used herein, the term "amino acid sequence" refers to an amino acid sequence of a naturally occurring or engineered protein molecule. "Amino acid sequence" and like terms, such as "polypeptide," "peptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

As used herein, the term "receptor proteins" and "membrane receptor proteins" refers to membrane spanning proteins that bind a ligand (*e.g.*, gp 130, a microbial molecule; endotoxin, such as LPS, LTA; dsRNA, and the like).

As used herein, the term "ligand" refers to a molecule that binds to a second molecule. A particular molecule may be referred to as either, or both, a ligand and second molecule. Examples of second molecules include a receptor of the ligand, and an antibody that binds to the ligand.

As used herein, the term "activating" when in reference to a biochemical response (such as kinase activity) and/or cellular response (such as cell proliferation) refers to increasing the biochemical and/or cellular response.

As used herein, the term "activated" when in reference to a cell, refers to a cell that has undergone a response that alters its physiology and shifts it towards making a biologically response and becoming biologically "active" hence "activated." For example, a monocyte becomes activated to mature into a macrophage. For another example, a macrophage becomes activated upon contact with endotoxin (such as LPS) wherein the activated macrophage can produce an increased level and/or type of molecule associated with activation (*e.g.* iNOS, MMP-12 Metalloelastase and the like). In another example, an immature dendritic cell becomes activated to mature into a functional dendritic cell. An "activated" cell does not necessarily, although it may, undergo growth or proliferation. Typically, activation of macrophages and DCs, unlike lymphocytes such as T-cells, B-cells and the like, does not stimulate proliferation. Activation can also induce cell death such as in activation-induced cell death (AICD) of T cells. In one embodiment o f the present invention, activation can lead towards apoptotic death.

As used herein, the terms "naturally occurring," "wild-type" and "wt" as used herein when applied to a molecule or composition (such as nucleotide sequence, amino acid sequence, cell, apoptotic blebs, external phosphatidylserine, *etc.*), mean that the molecule or composition can be found in nature and has not been intentionally modified by man. For example, a naturally occurring polypeptide sequence refers to a polypeptide sequence that is present in an organism that can be isolated from a source in nature, wherein the polypeptide sequence has not been intentionally modified by man.

The terms "derived from" and "established from" when made in reference to any cell disclosed herein refer to a cell which has been obtained (*e.g.*, isolated, purified, *etc.*) from the parent cell in issue using any manipulation, such as, without limitation, infection with virus, transfection with DNA sequences, treatment and/or mutagenesis using for example chemicals, radiation, *etc.*, selection (such as by serial culture) of any cell that is contained in cultured parent cells. A derived cell can be selected from a mixed population by virtue of response to a growth factor, cytokine, selected progression of cytokine treatments, adhesiveness, lack of adhesiveness, sorting procedure, and the like.

As used herein, the term "biologically active," refers to a molecules (*e.g.* peptide, nucleic acid sequence, carbohydrate molecule, organic or inorganic molecule, and the like) having structured, regulatory, and/or biochemical functions.

Unless defined otherwise in reference to the level of molecules and/or phenomena, the terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents when in reference to the level of any molecule (*e.g.*, protein, nucleic acid sequence, protein sequence, proliferation, rate of differentiation, *etc.*), phenomenon (*e.g.*, protein-protein interactions, catalytic activity, apoptosis, cell death, cell survival, cell proliferation, cell differentiation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) in a first sample relative to a second sample, mean that the quantity of molecule and/or phenomenon in the first sample is lower than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the reduction may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, difficulty in breathing, clarity of vision, nausea, tiredness, *etc.* In another embodiment, the quantity of molecule and/or phenomenon in the first sample is at least 10% lower than, at least 25% lower than, at least 50% lower than, at least 75%

lower than, and/or at least 90% lower than the quantity of the same molecule and/or phenomenon in a second sample.

As exemplified herein, in one embodiment, the quantity of substance and/or phenomenon in the first sample is at least 5% lower than the quantity of the same substance and/or phenomenon in a second sample. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 25% lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 50% lower than the quantity of the same substance and/or phenomenon in a second sample. In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 90% lower than the quantity of the same substance and/or phenomenon in a second sample. In one embodiment, the reduction may be determined subjectively, for example when comparing mRNA levels, *etc.*.

As used herein, the term "apoptosis" refers to the process of non-necrotic cell death that takes place in metazoan animal cells following activation of an intrinsic cell suicide program. Apoptosis is a normal process in the proper development and homeostasis of metazoan animals and usually leads to cell death. Apoptosis is also triggered pathologically by microbial infections resulting in increasing susceptibility to apoptosis and/or outright death. Apoptosis involves sequential characteristic morphological and biochemical changes. One early marker of apoptosis is the flipping of plasma membrane phosphatidylserine, inside to outside, with cellular blebbing called "zeiosis,"of plasma membrane releasing vesicles containing cellular material including RNA and DNA as apoptotic bodies. During apoptosis, there is cell expansion followed by shrinkage through release of apoptotic bodies and lysis of the cell, nuclear collapse and fragmentation of the nuclear chromatin, at certain intranucleosomal sites, due to activation of endogenous nucleases. Apoptotic bodies are

typically phagocytosed by other cells, in particular immunocytes such as monocytes, macrophages, immature dendritic cells and the like. One of skill in the art appreciates that reducing the ability to undergo apoptosis results in increased cell survival, without necessarily (although it may include) increasing cell proliferation. Accordingly, as used herein, the terms "reduce apoptosis" and "increase survival" are equivalent. Also, as used herein, the terms "increase apoptosis" and "reduced survival" are equivalent.

As used herein, the term "cellular response" refers to an increase or decrease of activity by a cell. For example, the "cellular response" may constitute but is not limited to apoptosis, death, DNA fragmentation, blebbing, proliferation, differentiation, adhesion, migration, DNA/RNA synthesis, gene transcription and translation, and/or cytokine secretion or cessation of such processes. A "cellular response" may comprise an increase or decrease of dephosphorylation, phosphorylation, calcium flux, target molecule cleavage, protein-protein interaction, nucleic acid-nucleic acid interaction, and/or protein/nucleic acid interaction and the like. As used herein, the term "target molecule cleavage" refers to the splitting of a molecule (for example in the process of apoptosis, cleavage of procaspases into fragments, cleavage of DNA into predicable sized fragments and the like). As used herein, the term "interaction" refers to the reciprocal action or influence of two or more molecules on each other.

As used herein, the term "transgenic" when used in reference to a cell refers to a cell which contains a transgene, or whose genome has been altered by the introduction of a transgene. The term "transgenic" when used in reference to a tissue or to a plant refers to a tissue or plant, respectively, which comprises one or more cells that contain a transgene, or whose genome has been altered by the introduction of a transgene. Transgenic cells, tissues and plants may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into a target cell or integration of the transgene into a chromosome of a target cell by way of human intervention, such as by the methods described herein.

As used herein, the term "transgene" as used herein refers to any nucleic acid sequence which is introduced into the cell by experimental manipulations. A transgene may be an "endogenous DNA sequence" or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA also includes an endogenous DNA sequence which contains some modification. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.*

As used herein, the terms "agent," "test agent," "molecule," "test molecule," "compound," and "test compound" as used interchangeably herein, refer to any type of molecule (for example, a peptide, nucleic acid, carbohydrate, lipid, organic molecule, and inorganic molecule, *etc.*) any combination molecule for example glycolipid, *etc.*) obtained from any source (for example, plant, animal, protist, and environmental source, *etc.*), or prepared by any method (for example, purification of naturally occurring molecules, chemical synthesis, and genetic engineering methods, *etc.*). Test agents are exemplified by, but not limited to individual and combinations of antibodies, nucleic acid sequences, and other agents as further described below.

In one embodiment, the term "test agent" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder

of bodily function. Test agents comprise both known and potential therapeutic agents. A test agent can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic agent" refers to a therapeutic agent that has been shown (*e.g.*, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention. In other words, a known therapeutic agent is not limited to an agent efficacious in the treatment of disease (*e.g.*, cancer). Agents are exemplified by, but not limited to, antibodies, nucleic acid sequences such as ribozyme sequences, and other agents as further described herein.

The test agents identified by and/or used in the invention's methods include any type of molecule (for example, a peptide, nucleic acid, carbohydrate, lipid, organic, and inorganic molecule, *etc.*) obtained from any source (for example, plant, animal, and environmental source, *etc.*), or prepared by any method (for example, purification of naturally occurring molecules, chemical synthesis, and genetic engineering methods, *etc.*).

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as used herein, are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters herein are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and without limiting the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters describing the broad scope of the invention are approximations, the numerical values in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains standard deviations that necessarily result from the errors found in the numerical value's testing measurements.

The term "not" when preceding, and made in reference to, any particularly named molecule (*e.g.*, nucleic acid sequence, protein sequence, apoptotic blebs, external

phosphatidylserine, *etc.*), and/or phenomenon (*e.g.*, apoptosis, cell death, cell survival, cell proliferation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) means that primarily the particularly named molecule or phenomenon is excluded.

The term "altering" and grammatical equivalents as used herein in reference to the level of any molecule (*e.g.*, nucleic acid sequence, protein sequence, apoptotic blebs, external phosphatidylserine, *etc.*), and/or phenomenon (*e.g.*, apoptosis, cell death, cell survival, cell proliferation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) refers to an increase and/or decrease in the quantity of the molecule and/or phenomenon, regardless of whether the quantity is determined objectively, and/or subjectively.

Unless defined otherwise in reference to the level of molecules and/or phenomena, the terms "increase," "elevate," "raise," and when in reference to the level of any molecule (*e.g.*, protein, nucleic acid sequence, protein sequence, proliferation, rate of differentiation, *etc.*), phenomenon (*e.g.*, protein-protein interactions, catalytic activity, apoptosis, cell death, cell survival, cell proliferation, cell differentiation, caspase cleavage, receptor dimerization, receptor complex formation, DNA fragmentation, molecule translocation, binding to a molecule, expression of a nucleic acid sequence, transcription of a nucleic acid sequence, enzyme activity, *etc.*) in a first sample relative to a second sample, mean that the quantity of the molecule and/or phenomenon in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the increase may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, difficulty in breathing, clarity of vision, nausea, tiredness, *etc.* In another embodiment, the quantity of the molecule and/or phenomenon in the first sample is at least 10% greater than, at least 25% greater than,

at least 50% greater than, at least 75% greater than, and/or at least 90% greater than the quantity of the same molecule and/or phenomenon in a second sample.

Reference herein to any specifically named protein (such as activin A polypeptide, KGF, *etc.*.) refers to any and all equivalent fragments, fusion proteins, and variants of the specifically named protein, having at least one of the biological activities (disclosed herein) of the specifically named protein, wherein the biological activity is detectable by any method.

The term "fragment" when in reference to a protein (such as activin A polypeptide, KGF, *etc.*.) refers to a portion of that protein that may range in size from four (4) contiguous amino acid residues to the entire amino acid sequence minus one amino acid residue. Thus, a polypeptide sequence comprising "at least a portion of an amino acid sequence" comprises from four (4) contiguous amino acid residues of the amino acid sequence to the entire amino acid sequence.

The term "fusion protein" refers to two or more polypeptides that are operably linked. The term "operably linked" when in reference to the relationship between nucleic acid sequences and/or amino acid sequences refers to linking the sequences such that they perform their intended function. For example, operably linking a promoter sequence to a nucleotide sequence of interest refers to linking the promoter sequence and the nucleotide sequence of interest in a manner such that the promoter sequence is capable of directing the transcription of the nucleotide sequence of interest and/or the synthesis of a polypeptide encoded by the nucleotide sequence of interest. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "variant" of a protein (such as activin A polypeptide, KGF, *etc.*) as used herein is defined as an amino acid sequence which differs by insertion, deletion, and/or conservative substitution of one or more amino acids from the protein of which it is a variant. The term "conservative substitution" of an amino acid refers to the replacement of that amino acid with another amino acid which has a similar hydrophobicity, polarity, and/or structure. For example, the following aliphatic amino acids with neutral side chains may be

conservatively substituted one for the other: glycine, alanine, valine, leucine, isoleucine, serine, and threonine. Aromatic amino acids with neutral side chains which may be conservatively substituted one for the other include phenylalanine, tyrosine, and tryptophan. Cysteine and methionine are sulphur-containing amino acids which may be conservatively substituted one for the other. Also, asparagine may be conservatively substituted for glutamine, and *vice versa*, since both amino acids are amides of dicarboxylic amino acids. In addition, aspartic acid (aspartate) may be conservatively substituted for glutamic acid (glutamate) as both are acidic, charged (hydrophilic) amino acids. Also, lysine, arginine, and histidine my be conservatively substituted one for the other since each is a basic, charged (hydrophilic) amino acid. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological and/or immunological activity may be found using computer programs well known in the art, for example, DNAStar™ software. In one embodiment, the sequence of the variant has at least 95% identity, at least 90% identity, at least 85% identity, at least 80% identity, at least 75% identity, at least 70% identity, and/or at least 65% identity with the sequence of the protein in issue.

Reference herein to any specifically named nucleotide sequence (such as a sequence encoding activin A polypeptide, KGF, *etc.*) includes within its scope any and all equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence, and that have at least one of the biological activities (such as those disclosed herein and/or known in the art) of the specifically named nucleotide sequence, wherein the biological activity is detectable by any method.

The "fragment" or "portion" may range in size from an exemplary 5, 10, 20, 50, or 100 contiguous nucleotide residues to the entire nucleic acid sequence minus one nucleic acid residue. Thus, a nucleic acid sequence comprising "at least a portion of" a nucleotide

sequence (such as sequences activin A polypeptide, KGF, etc.) comprises from five (5) contiguous nucleotide residues of the nucleotide sequence to the entire nucleotide sequence.

The term "homolog" of a specifically named nucleotide sequence refers to an oligonucleotide sequence which exhibits greater than 50% identity to the specifically named nucleotide sequence (such as a sequence encoding activin A polypeptide, KGF, *etc.*). Alternatively, or in addition, a homolog of a specifically named nucleotide sequence is defined as an oligonucleotide sequence which has at least 95% identity, at least 90% identity, at least 85% identity, at least 80% identity, at least 75% identity, at least 70% identity, and/or at least 65% identity to nucleotide sequence in issue.

With respect to sequences that hybridize under stringent conditions to the specifically named nucleotide sequence (such as a sequence encoding activin A polypeptide, KGF, *etc.*), high stringency conditions comprise conditions equivalent to binding or hybridization at 68°C in a solution containing 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution containing 0.1X SSPE, and 0.1% SDS at 68°C. "Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4-H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42°C.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization condition also results in hybridization of the first nucleic

acid sequence with the other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence.

As will be understood by those of skill in the art, it may be advantageous to produce a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence possesses non-naturally occurring codons. Therefore, in some embodiments, codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.*, Nucl. Acids Res., 17 (1989)) are selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

A "composition" comprising a particular polynucleotide sequence (such as a sequence encoding an activin A polypeptide, KGF, *etc.*) and/or comprising a particular protein sequence (such as activin A polypeptide, KGF, *etc.*) as used herein refers broadly to any composition containing the recited polynucleotide sequence (and/or its equivalent fragments, homologs, and sequences that hybridize under highly stringent and/or medium stringent conditions to the specifically named nucleotide sequence) and/or the recited protein sequence (and/or its equivalent fragments, fusion proteins, and variants), respectively. The composition may comprise an aqueous solution containing, for example, salts (*e.g.*, NaCl), detergents (*e.g.*, SDS), and other components (*e.g.*, Denhardt's solution, dry milk, salmon sperm DNA, *etc.*).

The invention does not limit the source (*e.g.*, cell type, tissue, animal, *etc.*), nature (*e.g.*, synthetic, recombinant, purified from cell extract, *etc.*), and/or sequence of the nucleotide sequence of interest and/or protein of interest. In one embodiment, the nucleotide sequence of interest and protein of interest include coding sequences of structural genes (*e.g.*, probe genes, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*).

DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for maintenance of the undifferentiated state and/or pluripotency in embryonic stem (ES) cells, and in particular, maintaining undifferentiated human embryonic stem cell lines, using culture medium enriched with Activin A and/or keratinocyte growth factor, without using fibroblast feeder layers or leukemia inhibitory factor.

Maintenance of the undifferentiated state and pluripotency in mouse ES requires the presence of mouse fibroblast feeder layers (mEFs) or activation of STAT3 with leukemia inhibitory factor (LIF) [1]. Human embryonic stem cell lines (hES) have only recently become available for research [2, 3], and the intracellular pathways for self renewal and differentiation are, at this time, largely unknown. Recently we [4] and others [5] have shown that STAT3 activation is not sufficient to block differentiation of human ES cell lines when grown on mEFs or when treated with conditioned media from mEFs. Here we show that culture medium enriched with Activin A is capable of maintaining hES in the undifferentiated state for 10 passages, without the need for feeder layers, conditioned medium from mEFs or STAT3 activation. hES cells retained markers of undifferentiated cells, including OCT-4, nanog and TRA-1-60, and remained pluripotent as shown by the *in vivo* formation of teratomas. The term "teratomas" refers to a tumor arising from cells of the three embryologic germ cell layers: ectoderm, mesoderm and endoderm. The term "embryologic germ cell layers" as used herein refers to layers of cells in the embryo that become specialized and express certain characteristic features in their final developed form (*e.g.* ectoderm, mesoderm and endoderm). The term "endoderm" refers to cells of the endodermal germ layers that develop into intestines. The term "mesoderm" refers to cells of the mesodermal germ layers that develop into blood vessels. The term "ectoderm" refers to cells of the ectodermal germ layers that develop into central and peripheral nerves, epidermis of skin.

The terms "stem cell," "unspecialized cell," "uncommitted cell" and "undifferentiated cell" refer to a cell that has a unique capacity to renew itself and to give rise to specialized

cell types that make up the tissues and organs of the body. A stem cell is without tissue-specific structures and tissue-specific functions (*e.g.* heart muscle cell, nerve cell, *e*tc.). Stem cells can be derived from embryo (*e.g.* embryonic stem cell), fetal and adult tissues. The terms "specialized," "committed," and "differentiated," refers to cells with tissue-specific structures and/or tissue-specific functions (*e.g.* heart muscle cell, nerve cell, *etc.*). The term "differentiation" when it refers a cell refers to the process whereby an unspecialized acquires the features of a specialized cell (*e.g.* a heart, liver, or muscle cell).

The term "progenitor cell" refers to a cell in fetal and/or adult tissues and is partially specialized, can divide, and gives rise to differentiated cells.

The terms "embryonic stem cell," "ES cells," and "pluripotent cell" refers to undifferentiated cells derived from the inner cell mass of embryos that have the potential to become any specialized cell type. The term "embryonic germ cell," refers to a cell derived of fetal tissue including, for example, from the primordial germ cells of the gonadal ridge of the 5 -to 10 week fetus. Later in development, the gonadal ridge develops into the testes or ovaries and the primordial germ cells give rise to eggs or sperm. Embryonic stem cells and embryonic germ cells are pluripotent but are not identical in properties.

The terms "mammalian embryonic stem cell" refers to ES cells derived from a mammal. It not meant to limit the mammals that can contribute stem cells and can include humans (hES), monkeys, great apes, pigs, horses, cows, sheep, dogs, cats, mice, rats, *etc.*.

The terms "adult stem cell," "multipotent stem cell," and "somatic stem cell" refer to an undifferentiated cell found in a differentiated tissue that can proliferate and differentiate to yield the specialized cell types of the tissue from which it originated.

The term "clonality derived stem cell" refers to a stem cell that is generated by the division of a single stem cell and is genetically identical to that stem cell.

The terms "totipotent," "pluripotent," "and "multipotent" refer to cells at different stages in development. The term "totipotent stem cells" refers to a cell that can form after the division of a fertilized egg and can form a blastocyst and develop into a complete

individual (*e.g.* mouse Oct-4+ cells). The inner layer of a blastocyst contains pluripotent stem cells. The term "pluripotent cells" refers to a cell that has the potential to develop into any cell type. The term "multipotent stem cells" refers to a cell that is found in mature tissue with the ability to differentiate into at least two or more differentiated descendant cells are formed by the body to replace worn out cells in tissues and organs (*e.g.* blood cells, *etc.*).

The term "plasticity" refers to the ability of stem cells from one adult tissue to generate a differentiated and specialized cell type (s) of another tissue.

The term "feeder layer" refers to cells used in co-culture for a desired effect, for example to maintain pluripotent stem cells.

Pluripotentiality of hES can only be maintained when grown on mEFs, [2, 3] conditioned medium from mEFs [6] or on human feeder layers [7]. In addition, the signals received from the feeder layers do not operate through the LIF/gp130 pathway [4, 5]. Therefore alternate pathways, triggered by the contact of hES cells to feeder layers and/or soluble factor(s) present in the conditioned media (CM), must be responsible for maintenance of pluripotency. As we have previously shown [4], under phase contrast microscope and histologically, growth and phenotypic characteristics of HSF6 are similar on feeder layers and in mEF CM when grown on laminin coated dishes. In this case, the cells grow in distinct undifferentiated colonies. Thus, soluble factors secreted by the feeder layers are instrumental in maintaining pluripotency. To determine what these factors might be we tested various combinations of growth factors, based on our experience in culturing human fetal pancreatic tissue. We used laminin 1 for adhesion, based on preliminary experiments, and the high levels of a6 b1 expression in hES cells [6].

The term "culture medium," "medium," "cell medium," and "cell growth medium" refers to the broth that covers cells in a vessel that contains nutrients to feed the cells as well as other growth factors that may be added to direct desired changes in the cells (*e.g.* cell division, inhibitor of differentiation, inducing differentiation, *etc.*).

The term "conditioned medium" refers to culture medium that has been in contact with live cells and thus contains a range of cell-derived molecules (*e.g.* growth substances, *etc.*) that when placed in contact with a subsequent batch of cells may enhance the growth or differentiation of subsequent cells. The term "non conditioned medium" refers to cell medium that has not been in contact with cells.

The term "fibroblast feeder" refers to a feeder layer comprising fibroblasts. The term "fibroblast" refers to a stellate (star-shaped) or spindle-shaped cell with cytoplasmic processes present in connective tissue, capable of forming fibers such as collagen fibers.

The term "mouse fibroblast feeder," and "mEF" refers to a feeder layer comprising mouse fibroblasts.

The term "conditioned medium from mouse fibroblast feeder" refers to culture medium that has been exposed to mouse fibroblast cells.

The term "laminin" and "LAM" refers to an extracellular matrix protein which contains a number of functional domains that allow it to assemble into sheets to act as a cell attachment substrate and allows it to act as a ligand to act as a growth factor (*e.g.* inducing differentiation, *etc.*).

Here we show that when hES cells were grown on laminin in the presence of Activin A and KGF they remained undifferentiated following continuous growth over 10 passages, staining uniformly for the stem cell markers TRA-1-60 and OCT-4 (Fig. 1a panel II), comparable to the staining for cells on feeder layers Fig.1a panel I or in CM (not shown). Robust gene expression of *oct-4*, *nanog* and *telomerase* was also observed by RT-PCR in the cell monolayers, with levels comparable to those obtained in colonies growing on feeder layers (fig.1b). Morphology gradually changed from the usual tight colony formation, to an irregular monolayer of uniformly shaped cells, that appeared larger than what is seen in the original colonies (fig 1a panel II). If allowed, cells eventually formed a continuous monolayer and mounded up in the dish. However these morphologic changes were reversible; when cells

were placed back on feeder layers they gradually resumed the colony formation similar to the expected morphology on feeder layers (fig. 1a panel III).

When Activin A was removed from the growth medium, the cell morphology rapidly changed to a more differentiated type (fig. 1a panel IV); after 1 week the cells no longer expressed nanog (fig1b), with concomitant loss of immunoreactive TRA-1-60 (fig. 1a panel IV) and reduced levels of OCT-4 protein (Fig.1a panel IV and message (fig1b). When Activin A was replaced with BMP-4, the cells were unable to maintain their undifferentiated phenotype, with loss of expression for nanog, oct-4 and telomerase.after 1 week (fig1b); when KGF was removed, the cells maintained their undifferentiated phenotype (fig1a panel V, and fig. 1b) but the proliferation rate decreased and they could not be subcultured beyond one passage.

The term "POU" is an acronym derived from the names of three mammalian transcription factors, the pituitary-specific Pit-1, the octamer-binding proteins Oct-1 and Oct-2, and the neural Unc-86 from Caenorhabditis elegans.

The term "POU transcription factor" refers to a member of a POU gene family that is a DNA-binding protein capable to activate the transcription of genes bearing cis-acting elements containing an octameric sequence called the octamer motif, within their promoter or enhancer region. The consensus-binding motif recognized by POU factors is the sequence ATGCAAAT that was originally found as an element controlling the immunoglobulin heavy chain enhancer

The terms "Oct-4 POU transcription factor" "octamer-binding transcription factor 3," "Oct-3," "OCT3," "Octamer-binding transcription factor 4" "Oct-4," "OCT4," "POU5F1," "OTF3," "class V POU factor Oct-3," "POU domain, class 5, transcription factor 1," refer to a POU transcription factor in undifferentiated stem cells.

The term "nanog" refers to a homeobox transcription factor found in undifferentiated stem cells.

The term "transcription factor" refers to a molecule that modulates expression of genes such as to regulate cell growth, differentiation and embryonic development.

The term "tumor rejection antigen," "TRA," "human embryonal carcinoma marker antigen," refer to keratin sulphate-associated antigens. These include proteins detected with monoclonal antibodies for TRA-1-60, TRA-1-81, TRA-1-85, TRA-2-54, TRA-2-49.

The term "stage-specific embryonic antigens," "SSEA-1," "SSEA-2, "SSEA-3," "SSEA-4" refer to a carbohydrate antigen whose cell surface expression changes upon differentiation. For example in murine ES cells, undifferentiated murine pluripotent cells express SSEA-1 while differentiation is characterized by the loss of SSEA-1 expression and may be accompanied, in some instances, by the appearance of SSEA-3 and SSEA-4. For example in humans, undifferentiated human EC, ES and EG cells express the antigens SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 while differentiating human EC and ES cells are characterized by an increase in SSEA-1 expression and a down regulation of SSEA-3 and SSEA-4.

Activin A, a member of the transforming growth factor ß family, was initially isolated from porcine follicular fluid [8, 9] as a stimulator of FSH synthesis and secretion. "Activin," "activin A," "activin B," "activin AB," "erythroid differentiation protein," and "EDF" are members of the TGF-beta-family that activates diverse biological functions such as hypothalamic and pituitary hormone secretion, gonadal hormone secretion, stem cell development and maturation, erythroid differentiation, insulin secretion, nerve cell survival, embryonic axial development, bone growth, *etc.*, wherein their function is related to their subunit composition. The term "activin A" and "ACTA," "ACTa," and "ACT" refers to a homodimer of "inhibin beta-A chain" or "activin beta-A chain." The term "activin B" refers to a homodimer of "inhibin beta-B chain" or a homodimer of "activin beta-B chain. The term "activin AB" refers to a dimer of "inhibin beta-A chain and beta-B chain. The terms "inhibin" and "inhibins" refers to a member of the TGF-beta-family that inhibits diverse biological functions such as hypothalamic and pituitary hormone secretion, gonadal hormone

secretion, stem cell development and maturation, erythroid differentiation, insulin secretion, nerve cell survival, embryonic axial development, bone growth, *etc.* and may oppose the functions of the activins, wherein their function is related to their subunit composition.

It has been identified in a wide variety of tissues as an autocrine or paracrine regulator of diverse biological functions (for review see [10]). Importantly, we found high expression of Activin A transcripts in mEFs and abundant secreted protein in the conditioned medium from mEFs (fig1c). Moreover, the HSF6 cells differentiated when grown on mEFs in the presence of follistatin, a natural inhibitor of Activin A [10] (fig.1a panel VI and fig 1b). Interestingly, Activin A has been shown to be secreted by other mesenchymal cells, and its secretion is upregulated by FGF2 [11], which is used routinely when culturing hES on mEFs. Taken together these data imply that Activin A, secreted by the mEFs is responsible for maintenance of "stemness" in hES cells.

The inhibition of differentiation in hES was specific for Activin A. While BMP-4, another TGF ß family member can maintain pluripotency in mouse ES cells [12], this is not the case with hES. This is not surprising since mES and hES also differ in their dependence on LIF for maintenance [4]. In mES cells BMP-4 plays a paradoxical role in both maintenance of pluripotency and differentiation [12, 13], most likely depending on other factors present or on stage of development. hES cells differentiated rapidly in the presence of BMP-4. and KGF, and expression of oct-4, nanog and telomerase was lost after 1 week culture.

Activin A has also been implicated in differentiation of mES into mesoderm [13], of human pancreatic precursor cells into ß cells [14], inhibition of neural differentiation [15, 16], and more recently induction of endoderm in hES cells [17], This, however, is the first documentation of an important role for Activin A in maintenance of stem cells in the undifferentiated state, and of its presence in conditioned medium from mEFs.

Teratomas grown in nude mice after transplantation of hES grown in monolayer in the presence of Activin A and KGF (figure 2a,) showed many ectodermal, endodermal and mesodermal structures. In addition, rtpcr performed on RNA from embryoid bodies showed

gene expression specific for all 3 embryonic cell layers (fig2b). These data show that maintenance of hES in medium containing Activin A allows the maintenance of pluripotentiality without the need for coculture with other foreign or human cells.

The terms "keratinocyte growth factor," "KGF," "Fibroblast growth factor-7," "FGF-7," "HBGF-7," "heparin-binding growth factor-7," refers to a growth factor of the fibroblast growth factor family active on keratinocytes.

The term "keratinocyte," refers to a cell that makes keratin. The term "keratin" refers to large molecules found in specialized epithelial cells such as those of the upper layer of the skin, hair, nails, and animal horns.

The term "leukemia inhibitory factor," "LIF, "leukemia inhibitory factor precursor ," "LIF," "differentiation-stimulating factor," "D factor," "melanoma-derived LPL inhibitor," "MLPLI," "HILDA" "human interleukin for DA cells" "myeloid growth factor human interleukin for DA cells" refers to a cell messenger protein that inhibits differentiation of stem cells and induces terminal differentiation in leukemic cells and induces hematopoietic differentiation in normal and myeloid leukemia cells.

The term "embryo" refers to a developing organism from the time of fertilization until significant differentiation has occurred, for example in humans until the end of the eighth week of gestation, when it becomes known as a fetus.

The term "karyotype" refers to the chromosome characteristics, (*e.g.* the number, shape, *etc.*) of an individual cell or cell line, usually presented as a set of photographed, banded chromosomes arranged in order from largest to smallest.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques. The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule.

The term "cell surface molecules" refers to cell molecules at least a portion of which is attached to, and/or spans, the plasma membrane. In a preferred embodiment, at least a portion

of the cell surface molecule is located on the extracellular side of the plasma membrane, such that it is accessible to molecules outside the cell. For example, a polypeptide that is expressed (*e.g.*, by the wild type cells and/or by genetic engineering) on the surface of muscle stem cells but not other cells of a cell population serves as a marker protein for the muscle stem cells. Cell surface molecules include "cell surface marker molecules", such as an antigen to which an antibody specifically binds (*e.g.*, in cell sorting methods to produce a population of cells enriched for cells that express the marker molecule).

The terms "STAT," and "Signal Transducers and Activators of Transcription," refer to a molecule in the family of proteins that regulates genes (*e.g.* STAT1, STAT2, STAT3, STAT4, STAT5. STAT6, *etc.*). The term "STAT3" refers to an oncogene since involved in activating expression of cyclin D1, c-Myc, bcl-xl, *etc.*, and involved in promoting cell-cycle progression, cellular transformation, and in preventing apoptosis.

As used herein, the term "protein kinase" refers to a protein that catalyzes the addition of a phosphate group from a nucleoside triphosphate to an amino acid in a protein. Kinases comprise the largest known enzyme superfamily and vary widely in their target proteins. Kinases can be categorized as protein tyrosine kinases (PTKs), which phosphorylate tyrosine residues (*e.g.* Janus family tyrosine kinases (JAK)), and protein serine/threonine kinases (STKs), which phosphorylate serine and/or threonine residues and the like.

Non-transmembrane PTKs form signaling complexes with the cytosolic domains of plasma membrane receptors. Receptors that signal through non-transmembrane PTKs include cytokine (*e.g.* glycoprotein 130 (gp130) family), hormone, and antigen-specific lymphocytic receptors. Many PTKs were first identified as oncogene products in cancer cells in which PTK activation was no longer subject to normal cellular controls. In fact, about one third of the known oncogenes encode PTKs. Furthermore, cellular transformation (oncogenesis) is often accompanied by increased tyrosine phosphorylation activity (See, *e.g.*, Carbonneau, H. and Tonks, Annu. Rev. Cell Biol. 8:463-93, 1992). Regulation of PTK activity may therefore be an important strategy in controlling some types of cancer.

Examples of protein kinases include, but are not limited to, cAMP-dependent protein kinase, protein kinase C, and cyclin-dependent protein kinases (See, *e.g.*, U.S. Pat. Nos. 6,034,228; 6,030,822; 6,030,788; 6,020,306; 6,013,455; 6,013,464; and 6,015,807, all of which are incorporated herein by reference). Examples of targets for kinases are STATs.

As used herein, the term "protein phosphatase" refers to proteins that remove a phosphate group from a protein. Protein phosphatases are generally divided into two groups, receptor-type and non-receptor type (*e.g.* intracellular) proteins. An additional group includes dual specificity phosphatases. Most receptor-type protein tyrosine phosphatases contain two conserved catalytic domains, each of which encompasses a segment of 240 amino acid residues (See *e.g.*, Saito *et al.* Cell Growth and Diff. 2:59, 1991). Receptor protein tyrosine phosphatases can be subclassified further based upon the amino acid sequence diversity of their extracellular domains (See *e.g.*, Krueger *et al.* Proc. Natl. Acad. Sci. USA 89:7417-7421, 1992). Examples of protein phosphatases include, but are not limited to, human protein phosphatase (PROPHO), FIN13, cdc25 tyrosine phosphatase, protein tyrosine phosphatase (PTP) 20, PTP 1D, PTP-D1, PTP .lambda., PTP-S31 (See *e.g.*, U.S. Pat. Nos. 5,853,997; 5,976,853; 5,294,538; 6,004,791; 5,589,375; 5,955,592; 5,958,719; and 5,952,212; all of which are incorporated herein by reference). Examples of targets for protein phosphatase are STATs.

As used herein, the term "phosphorylation" refers to the addition of phosphate groups. Protein phosphorylation is catalyzed by protein kinases which attach phosphate groups to hydroxyls of Ser, Thr and/or Tyr side chains. As used herein, the term "dephosphorylation" refers to the removal of a phosphate group. Protein dephosphorylation is catalyzed by protein phosphatases which remove phosphate groups from the side chains of Ser, Thr, and/or Tyr.

In conclusion, the identification of Activin A as a key factor in mediating these cellular events will help to unravel the biochemical pathways responsible for “stemness”, and this method of culture will lead to increased efficiency in generating enough cells for clinical applications. These findings are timely, given the recent report of derivations of 17 new stem

cell lines with non NIH funding [18]. It is to be hoped that this simplified culture method will also aid in derivation of new human embryonic cell lines without feeder layers.

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EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. In the experimental disclosure which follows, the following abbreviations apply: M (molar); mM (millimolar); μM (micromolar); nM (nanomolar); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams); μg (micrograms); pg (picograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); °C (degrees Centigrade).

EXAMPLE 1

Materials and Methods The following is a description of exemplary materials and methods that were used in subsequent Examples.

Stem cell culture hES cell line HSF6 was maintained on mitomycin C treated CF1 mouse feeder layers (mEF) at 37^{0}C, 5% CO_2 in DSR medium, which consisted of high glucose DMEM containing knockout serum replacer, glutamine, non essential amino acids, 0.1mM ß-mercaptoethanol (all from Gibco, Carlsbad, CA; http://www.invitrogen.com) as previously described [19], or on laminin (20 μg/ml, Chemicon, www.chemicon.com) coated dishes or in DSR containing 50 ng/ml human recombinant Activin A (ACT A), 50 ng/ml human recombinant Keratinocyte Growth Factor (KGF), both from Preprotech Inc. (Rocky Hill NJ www.preprotech.com <http://www.preprotech.com>). These concentrations were determined from previous experiments with human fetal pancreatic cell culture [14, 20]. In some experiments 10 ng/ml human recombinant Bone Morphogenetic Protein 4 (BMP-4; R&D Systems

Minneapolis, MN. www.RnDSystems.com) was used to replace ACT A, and 0.4 μg/ml follistatin (R&D Systems) was added to ES cells grown on mEFs, sufficient to neutralize 50 ng/ml Act A according to manufacturer's directions. Medium was changed every day on cells grown on mEFs and every other day,, on cells grown on laminin with the growth factors. Cells were passaged weekly at 1:3 or 1:4 dilution.

Immunohistochemistry Stem cell cultures were grown on coverslips coated with mEFs or laminin fixed with 4% paraformaldehyde and stained. Protein expression of the stem cell markers TRA-1-60 and Oct-4 was analyzed by immunohistochemistry using primary mouse anti-TRA-1-60 (Chemicon) and rabbit anti-OCT-4 antiserum (a generous gift from Dr Hans Scholer, U Penn). Control slides were incubated with mouse IgM and rabbit IgG. Affinity purified rhodamine red-conjugated donkey anti-mouse IgM and fluorescein-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) were directed against primary antibodies. Coverslips were mounted in anti fade medium (Biomeda Corp, Foster City, CA; http://biomeda.com) and viewed on a Nikon eclipse E800 microscope (NikonUSA, Melville, NY; http://www.nikonusa.com) equipped with a fluorescent attachment. Images were captured with a SPOT digital camera (Diagnostic Instruments Inc, Sterling Heights, MI; http://www.diaginc.com) and acquired through Image Pro Plus 4.0 (Media Cybernetics, Silver Spring, MD; http://www.mediacy.com). Color composite pictures were processed using Adobe Photoshop 7.0 (Adobe Systems, Mountainview, CA; http://www.adobe.com).

RT-PCR. RNA was purified using the RNeasy minikit including DNase treatment (Qiagen, Valencia, CA; http://www1.qiagen.com) and reverse transcribed using AMV with 3.2μg of random primer (both Roche, Indianapolis, IN; http://www.roche-applied-science.com) and 1μg of total RNA in a reaction volume of 20μL. 1μL of cDNA was used for each PCR reaction in a total volume of 50μL. ß-actin expression was used for sample -----Probes were prepared specific for human oct-4, nanog, and telomerase. Oligonucletide primers had the following

sequences: ß-actin cgcaccactggcattgtcat forward, ttctccttgatgtcacgcac reverse, oct-4 gagcaaaacccggaggagt forward, ttctctttcgggcctgcac reverse, nanog gcttgccttgctttgaagca, forward, ttcttgactgggaccttgtc reverse, telomerase. The PCR product was loaded onto a 1.2% agarose gel and stained with ethidium bromide.

Pluripotency was assessed *in vivo* by examining teratoma formation 8 weeks after transplanting the hES cells under the renal capsule of nude mice as previously described for analysis of pancreatic progenitor islet cell differentiation [21]. Briefly hES were removed from laminin or mEFs, and allowed to form embryoid bodies overnight in Costar Ultra Low Cluster dishes (Corning Inc, Corning NY, www.corning.com). They were centrifuged into a pellet, collected in a 10 µl positive pressure pipet and carefully inserted under the renal capsule. This method has been highly successful in experimental islet transplantation, and is also very efficient for analysis of teratoma formation from hES cells. Grafts were removed, fixed and stained with hematoxylin and eosin. Pluripotency was assessed *in vitro* by assessing gene expression following embryoid body formation as described.

EXAMPLE 2

Figure 1a. Morphology and differentiation state of HSF6 cells observed by phase contrast microscopy (upper layer), and immunohistochemistry (lower layer). For immunhistochemical analysis, antibodies against the human stem cell markers TRA-1-60 (red, cytoplasmic) and OCT-4 (green, nuclear) were used. Panel I: HSF6 cells cultured on mEFs show typical colony formation, with uniform staining for stem cell markers. Panel II: HSF6 cultured on laminin in the presence of ACT and KGF for several weeks, grow as irregular monolayers, with larger cell size than when grown on mEFs, but show robust staining for TRA-1-60 and Oct4, proof of their undifferentiated state. Panel III: cells from panel II, when put back on mEFs resume colony morphology after 1 week. Panel IV: cells from panel II, grown in the absence of ACT for 1 week, show distinct change in morphology and phenotype, with no

staining for TRA-1-60 and very little staining for OCT-4, indicating differentiation. Panel V: cells from panel II grown in the absence of KGF for 1 week, show no change in phenotype, however proliferation was reduced and they could not be passaged further. Panel VI: HSF6 cells from panel I, cultured on mEFs in the presence of follistatin for 1 week, showed olony morphologic changes and lost staining for TRA-160, with reduced staining for OCT-4, indicating differentiation. MEF = mouse embryonic fibroblasts, LAM=laminin, ACT = Activin A, KGF= keratinocyte growth factor, FOL= Follistatin. Magnification bar = 100 μM

Figure 1b. Semi-quantitative RT-PCR of hES cells for oct-4, nanog and telomerase under a variety of culture conditions on mEFs (lane 1 and 6), or on laminin (lane 2-5). Cells differentiated on laminin in the absence of activin (lane 3, 5), and on mEFs in the presence of follistatin (lane 6), indicating the need for activin to maintain the undifferentiated phenotype. MEF=mouse feeder layers, ACT=Activin A, BMP=BMP-4, FOL = follistatin +/-: reverse transcriptase.

Figure 1c. Expression of activin in mEFs using RT-PCR and western blots. +/-: reverse transcriptase.

EXAMPLE 2

Figure 2a. Teratoma formation in nude mice. HSF6 cells cultured on laminin in the presence of ACT A and KGF for 4 weeks were transplanted under the renal capsule of nude mice. After 8 weeks, kidneys were removed, and teratomas showing evidence of all 3 cell layers were observed. MK= mouse kidney, B=bone, C=chondrocytes (both mesoderm), G= glial tissue (ectoderm), SE= secretory epithelium (endoderm). Magnification bar = 100 μM

Figure 2b. RT-PCR for embryoid bodies.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed

should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the invention.

Activin A maintains pluripotentiality of human embryonic stem cells in the absence of feeder layers.

Gillian M Beattie, Ana D Lopez, Nathan Bucay, Charles H King and Alberto Hayek

Maintenance of pluripotency in mouse ES requires the presence of mouse fibroblast feeder layers (mEFs) or activation of STAT3 with leukemia inhibitory factor (LIF) 1. In human embryonic stem cell lines (hES), the intracellular pathways for self renewal and differentiation are largely unknown 2, 3. Recently it has been shown that that STAT3 activation is not sufficient to block differentiation of hES cells when grown on mEFs or when treated with conditioned media from mEFs 4, 5. Here we show that Activin A is secreted by mEFs and that culture medium enriched with Activin A is capable of maintaining hES in the undifferentiated state for <10 passages, without the need for feeder layers, conditioned medium from mEFs or STAT3 activation. hES cells retained markers of undifferentiated cells, including OCT-4, nanog and TRA-1-60, and remained pluripotent as shown by the in vivo formation of teratomas.

Pluripotentaility of hES can only be maintained when grown on mEFs, 2, 3 conditioned medium from mEFs 6 or on human feeder layers 7,; In addition, the signals received from the feeder layers do not operate through the LIF/gp130 pathway 4, 5. Therefore alternate pathways, triggered by the contact of hES cells to feeder layers and/or soluble factor(s) present in the conditioned media (CM), must be responsible for maintenance of pluripotency. As we have previously shown 4, under phase contrast microscope and histologically, growth and phenotypic characteristics of HSF6 are similar on feeder layers and in mEF CM when grown on laminin coated dishes. In this case, the cells grow in distinct undifferentiated colonies. Thus, soluble factors secreted by the feeder layers are instrumental in maintaining pluripotency. To determine what these factors might be we tested various combinations of growth factors, based on our experience in culturing human fetal pancreatic tissue. We used

laminin 1 for adhesion, based on preliminary experiments, and the high levels of a6 b1 expression in hES cells 6. Here we show that when hES cells were grown on laminin in the presence of Activin A and KGF they remained undifferentiated following continuous growth over 10 passages, staining uniformly for the stem cell markers TRA-1-60 and OCT-4 (Fig. 1a panel II), comparable to the staining for cells on feeder layers Fig.1a panel I or in CM (not shown). Robust gene expression of oct-4, nanog and telomerase was also observed by RTPCR in the cell monolayers, with levels comparable to those obtained in colonies growing on feeder layers (fig.1b). Morphology gradually changed from the usual tight colony formation, to an irregular monolayer of uniformly shaped cells, that appeared larger than what is seen in the original colonies (fig 1a panel II). If allowed, cells eventually formed a continuous monolayer and mounded up in the dish. However these morphologic changes were reversible; when cells were placed back on feeder layers they gradually resumed the colony formation similar to the expected morphology on feeder layers (fig. 1a panel III).

When Activin A was removed from the growth medium, the cell morphology rapidly changed to a more differentiated type (fig. 1a panel IV); after 1 week the cells no longer expressed nanog (fig1b), with concomitant loss of immunoreactive TRA-1-60 (fig. 1a panel IV) and reduced levels of OCT-4 protein (Fig.1a panel IV and message (fig1b). When Activin A was replaced with BMP-4, the cells were unable to maintain their undifferentiated phenotype, with loss of expression for nanog, and oct-4.after 1 week (fig1b); when KGF was removed, the cells maintained their undifferentiated phenotype (fig1a panel V, and fig. 1b) but the proliferation rate decreased and they could not be subcultured beyond one passage.

Activin A, a member of the transforming growth factor ß family, was initially isolated from porcine follicular fluid 8, 9 as a stimulator of FSH synthesis and secretion. It has been identified in a wide variety of tissues as an autocrine or paracrine regulator of diverse biological functions (for review see 10). Importantly, we found high expression of Activin A transcripts in mEFs and abundant secreted protein in the conditioned medium from mEFs

(figlc). Moreover, the HSF6 cells differentiated when grown on mEFs in the presence of follistatin, a natural inhibitor of Activin A 10 (fig.1a panel VI and fig 1b). Interestingly, Activin A has been shown to be secreted by other mesenchymal cells, and its secretion is upregulated by FGF2 11, which is used routinely when culturing hES on mEFs. Taken together these data imply that Activin A, secreted by the mEFs is responsible for maintenance of "stemness" in hES cells.

The inhibition of differentiation in hES was specific for Activin A. While BMP-4, another TGF ß family member can maintain pluripotency in mouse ES cells 12, this is not the case with hES.. This is not surprising since mES and hES also differ in their dependence on LIF for maintenance 4. In mES cells BMP-4 plays a paradoxical role in both maintenance of pluripotency and differentiation 12, 13, most likely depending on other factors present or on stage of development. hES cells differentiated rapidly in the presence of BMP-4. and KGF, and expression of oct-4 and nanog was lost after 1 week culture.

Activin A has also been implicated in differentiation of mES into mesoderm 13, of human pancreatic precursor cells into ß cells 14, inhibition of neural differentiation 15, 16, and more recently induction of endoderm in hES cells 17, This, however, is the first documentation of an important role for Activin A in maintenance of stem cells in the undifferentiated state, and of its presence in conditioned medium from mEFs.

Teratomas grown in nude mice after transplantation of hES grown in monolayer in the presence of Activin A and KGF (figure 2a,) showed many ectodermal, endodermal and mesodermal structures. In addition, rtpcr performed on RNA from embryoid bodies showed gene expression specific for all 3 embryonic cell layers (fig2b) (NEED DATA this weekend). These data show that maintenance of hES in medium containing Activin A allows the maintenance of pluripotentiality without the need for coculture with other foreign or human cells.

In conclusion, the identification of Activin A as a key factor in mediating these cellular events will help to unravel the biochemical pathways responsible for "stemness". An increased efficiency in the generation an culture of stem cells for potential clinical applications is timely, given the recent report of 17 newly derived stem cell lines available for non-federal supported research 18. This simplified culture method will facilitate the derivation of new human embryonic cell lines without the use of animal or human feeder layers.

Methods

Stem cell culture hES cell line HSF6 was maintained on mitomycin C treated CF1 mouse feeder layers (mEF) at 370C, 5% CO2 in DSR medium, which consisted of high glucose DMEM containing knockout serum replacer, glutamine, non essential amino acids, 0.1mM ß-mercaptoethanol (all from Gibco, Carlsbad, CA; http://www.invitrogen.com) as previously described 19, or on laminin (20 μg/ml, Chemicon, www.chemicon.com) coated dishes or in DSR containing 50 ng/ml human recombinant Activin A (ACT A), 50 ng/ml human recombinant Keratinocyte Growth Factor (KGF), both from Preprotech Inc. (Rocky Hill NJ www.preprotech.com). These concentrations were determined from previous experiments with human fetal pancreatic cell culture 14, 20. In some experiments 10 ng/ml human recombinant Bone Morphogenetic Protein 4 (BMP-4; R&D Systems Minneapolis, MN. www.RnDSystems.com) was used to replace ACT A, and 0.4 μg/ml follistatin (R&D Systems) was added to ES cells grown on mEFs, sufficient to neutralize 50 ng/ml Act A according to manufacturer's directions. Medium was changed every day on cells grown on mEFs and every other day, on cells grown on laminin with the growth factors. Cells were passaged weekly at 1:3 or 1:4 dilution.

Immunohistochemistry Stem cell cultures were grown on coverslips coated with mEFs or laminin fixed with 4% paraformaldehyde and stained. Protein expression of the stem cell markers TRA-1-60 and Oct-4 was analyzed by immunohistochemistry using primary mouse anti-TRA-1-60 (Chemicon) and rabbit anti-OCT-4 antiserum (a generous gift from Dr Hans

Scholer, U Penn). Control slides were incubated with mouse IgM and rabbit IgG. Affinity purified rhodamine red-conjugated donkey anti-mouse IgM and fluorescein-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) were directed against primary antibodies. Coverslips were mounted in anti fade medium (Biomeda Corp, Foster City, CA; http://biomeda.com) and viewed on a Nikon eclipse E800 microscope (NikonUSA, Melville, NY; http://www.nikonusa.com) equipped with a fluorescent attachment. Images were captured with a SPOT digital camera (Diagnostic Instruments Inc, Sterling Heights, MI; http://www.diaginc.com) and acquired through Image Pro Plus 4.0 (Media Cybernetics, Silver Spring, MD; http://www.mediacy.com). Color composite pictures were processed using Adobe Photoshop 7.0 (Adobe Systems, Mountainview, CA; http://www.adobe.com).

RT-PCR. RNA was purified using the RNeasy minikit including DNase treatment (Qiagen, Valencia, CA; http://www1.qiagen.com) and reverse transcribed using AMV with 3.2μg of random primer (both Roche, Indianapolis, IN; http://www.roche-applied-science.com) and 1μg of total RNA in a reaction volume of 20μL. 1μL of cDNA was used for each PCR reaction in a total volume of 50μL. Specific primers were designed for human oct-4, nanog , activin A and ß-actin; the latter was used as an internal control. Primers had the following sequences: oct-4 51-GAGCAAAACCCGGAGGAGT forward, TTCTCTTTCGGGCCTGCAC-31 reverse, nanog 51GCTTGCCTTGCTTTGAAGCA, forward, TTCTTGACTGGGACCTTGTC-31 reverse. Activin A 51-CTTGAAGAAGAGACCCGAT forward, CTTCTGCACGCTCCACTAC -31 reverse, ß-actin 51-CGCACCACTGGCATTGTCAT forward, TTCTCCTTGATGTCACGCAC-31 reverse, The PCR products were loaded onto a 1.2% agarose gel and stained with ethidium bromide.

Pluripotency was assessed in vivo by examining teratoma formation 8 weeks after transplanting the hES cells under the renal capsule of nude mice as previously described for

analysis of pancreatic progenitor islet cell differentiation 21. Briefly hES were removed from laminin or mEFs, and allowed to form embryoid bodies overnight in Costar Ultra Low Cluster dishes (Corning Inc, Corning NY, www.corning.com). They were centrifuged into a pellet, collected in a 10 µl positive pressure pipet and carefully inserted under the renal capsule. This method has been highly successful in experimental islet transplantation, and is also very efficient for analysis of teratoma formation from hES cells. Grafts were removed, fixed and stained with hematoxylin and eosin. Pluripotency was assessed in vitro by assessing gene expression following embryoid body formation as described .

Accession numbers

ß-actin GI: 33878222, oct-4 GI: 22056734, nanog GI: 13376297, activin A GI:31566358

Acknowledgements

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Figure Legends

Figure 1 Differentiation of hES cells in the absence of Activin A

a. Morphology and differentiation state of HSF6 cells observed by phase contrast microscopy (upper layer), and immunohistochemistry (lower layer). For immunhistochemical analysis, antibodies against the human stem cell markers TRA-1-60 (red, cytoplasmic) and OCT-4 (green, nuclear)were used. Panel I: HSF6 cells cultured on mEFs show typical colony formation, with uniform staining for stem cell markers. Panel II: HSF6 cultured on laminin in the presence of ACT and KGF for several weeks, grow as irregular monolayers,, with larger cell size than when grown on mEFs, but show robust staining for TRA-1-60 and Oct4, proof of their undifferentiated state. Panel III,: cells from panel II, when put back on mEFs resume colony morphology after 1 week. Panel IV; cells from panel II, grown in the absence of ACT for 1 week, show distinct change in morphology and phenotype, with no staining for TRA-1-60 and very little staining for OCT-4, indicating differentiation. Panel V: cells from panel II grown in the absence of KGF for 1 week, show no change in phenotype,:

however proliferation was reduced and they could not be passaged further. Panel VI: HSF6 cells from panel I, cultured on mEFs in the presence of follistatin for 1 week, showed colony morphologic changes and lost staining for TRA-160, with reduced staining for OCT-4, indicating differentiation. MEF = mouse embryonic fibroblasts, LAM=laminin, ACT = Activin A, KGF= keratinocyte growth factor, FOL= Follistatin. Magnification bar = 100 μM

b. Semi-quantitative RT-PCR of hES cells for oct-4 and nanog under a variety of culture conditions on mEFs (lane 1 and 6), or on laminin (lane 2-5). Expression of stem cell markers was lost in cells cultured for 1 week on laminin in the absence of activin (lane 3, 5), and reduced in cells cultured on mEFs after 1 week in the presence of follistatin (lane 6), indicating the need for activin to maintain the undifferentiated phenotype. MEF=mouse feeder layers, ACT=Activin A, BMP=BMP-4, FOL = follistatin +/-: reverse transcriptase.

c. Expression of activin in mEFs derived from CF-1 mice using RT-PCR and western blots. PCR product size is 262 bp; +/-: reverse transcriptase.

Figure 2 Maintenance of pluripotency in hES cells cultured with Activin A

a . Teratoma formation in nude mice. HSF6 cells cultured on laminin in the presence of ACT A and KGF for 4 weeks were transplanted under the renal capsule of nude mice. After 8 weeks, kidneys were removed, and teratomas showing evidence of all 3 cell layers were observed. MK= mouse kidney, B=bone, C=chondrocytes (both mesoderm), G= glial tissue (ectoderm), SE= secretory epithelium (endoderm). Magnification bar = 100 μM

b. RTPCR for embryoid bodies

ABSTRACT

The present invention relates to compositions and methods for maintenance of the undifferentiated state and/or pluripotency in embryonic stem (ES) cells, and in particular, maintaining undifferentiated human embryonic stem cell lines, using culture medium enriched with Activin A and/or keratinocyte growth factor, without using fibroblast feeder layers or leukemia inhibitory factor.

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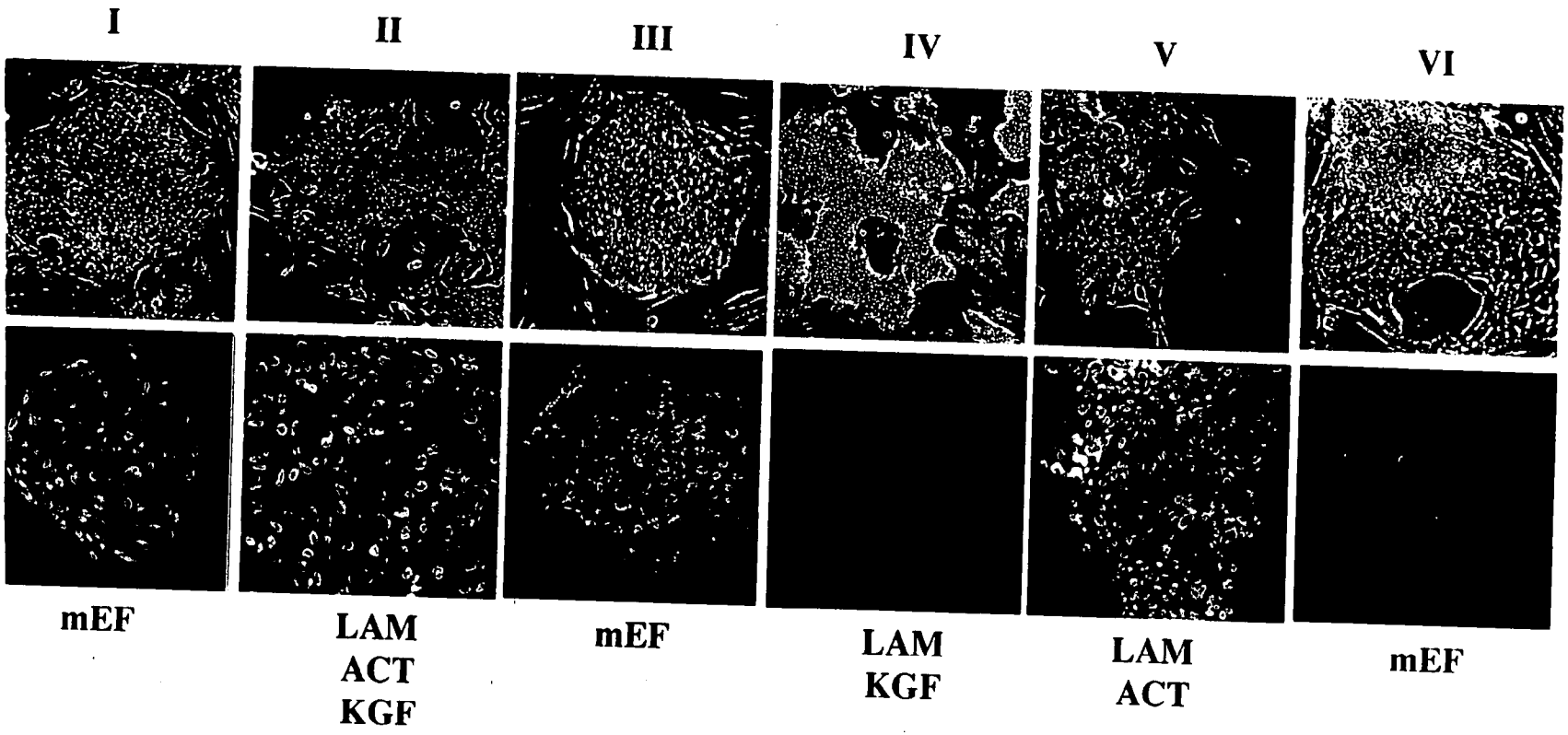

Fig. 1a

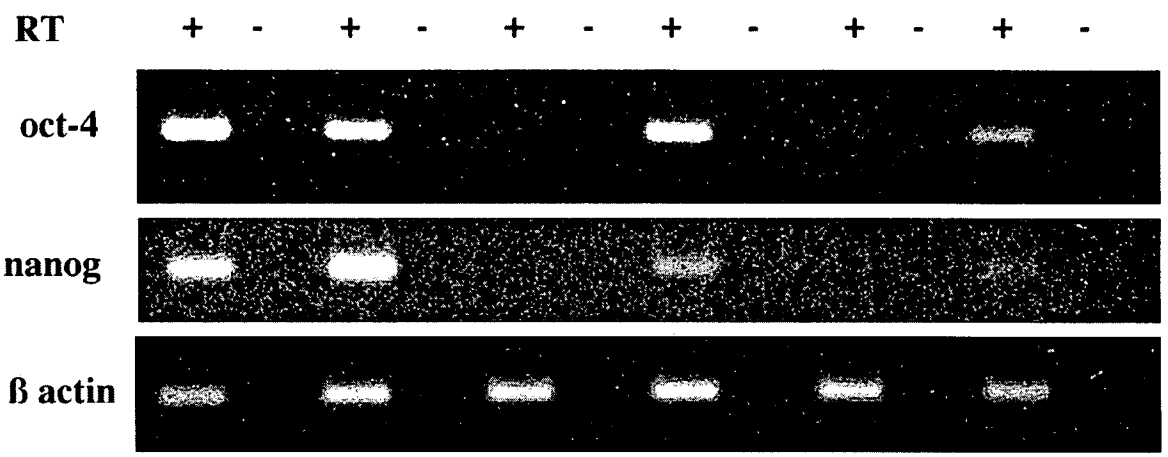

Fig. 1b

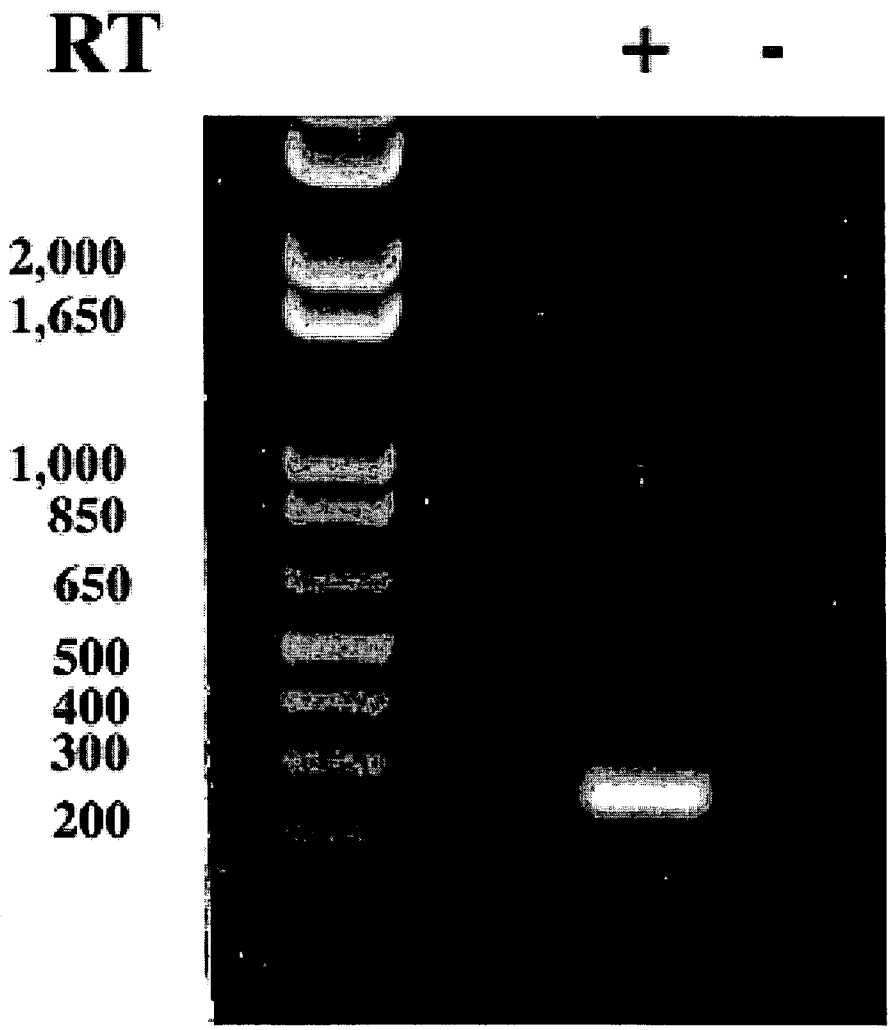

Fig. 1c

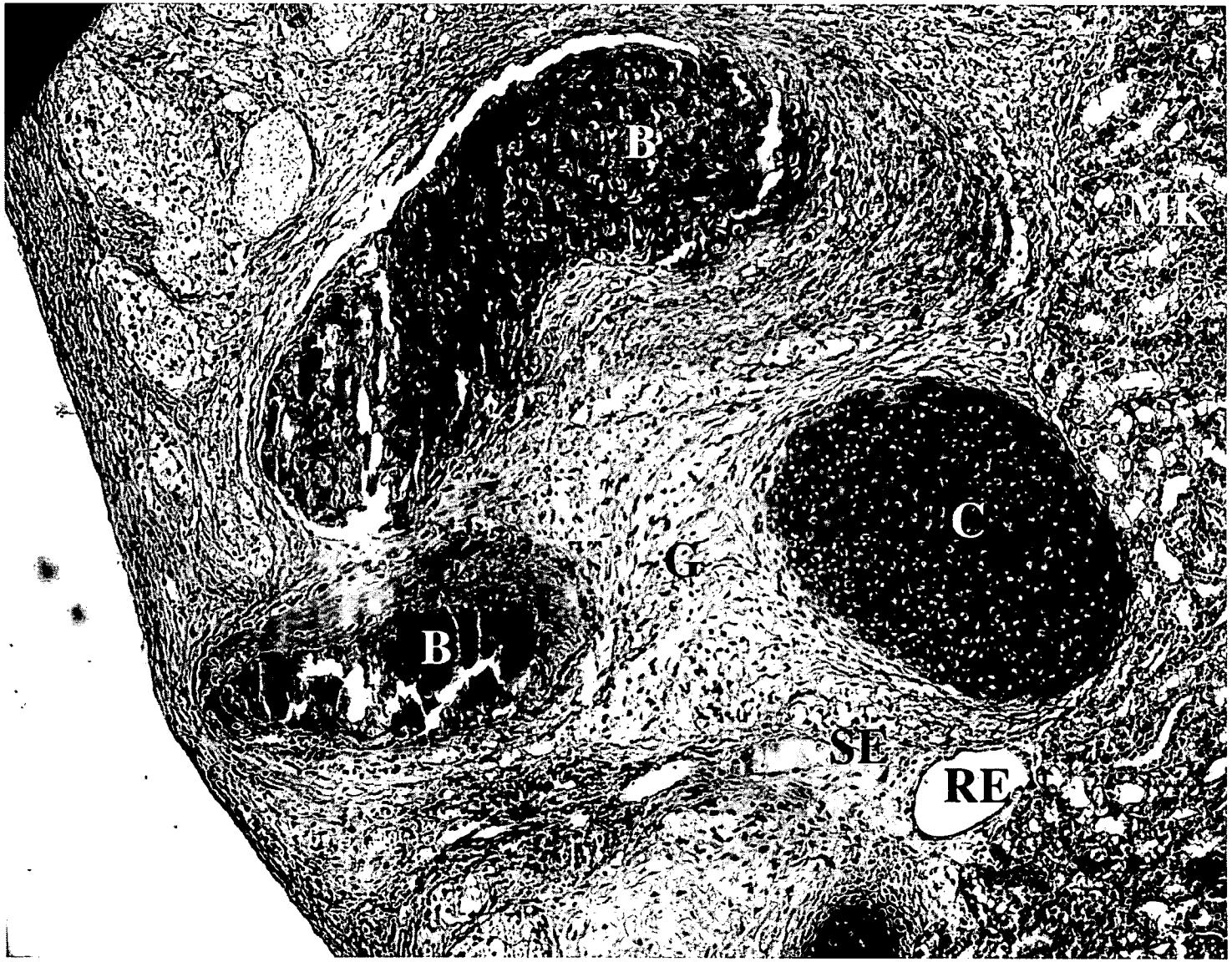

Fig. 2a

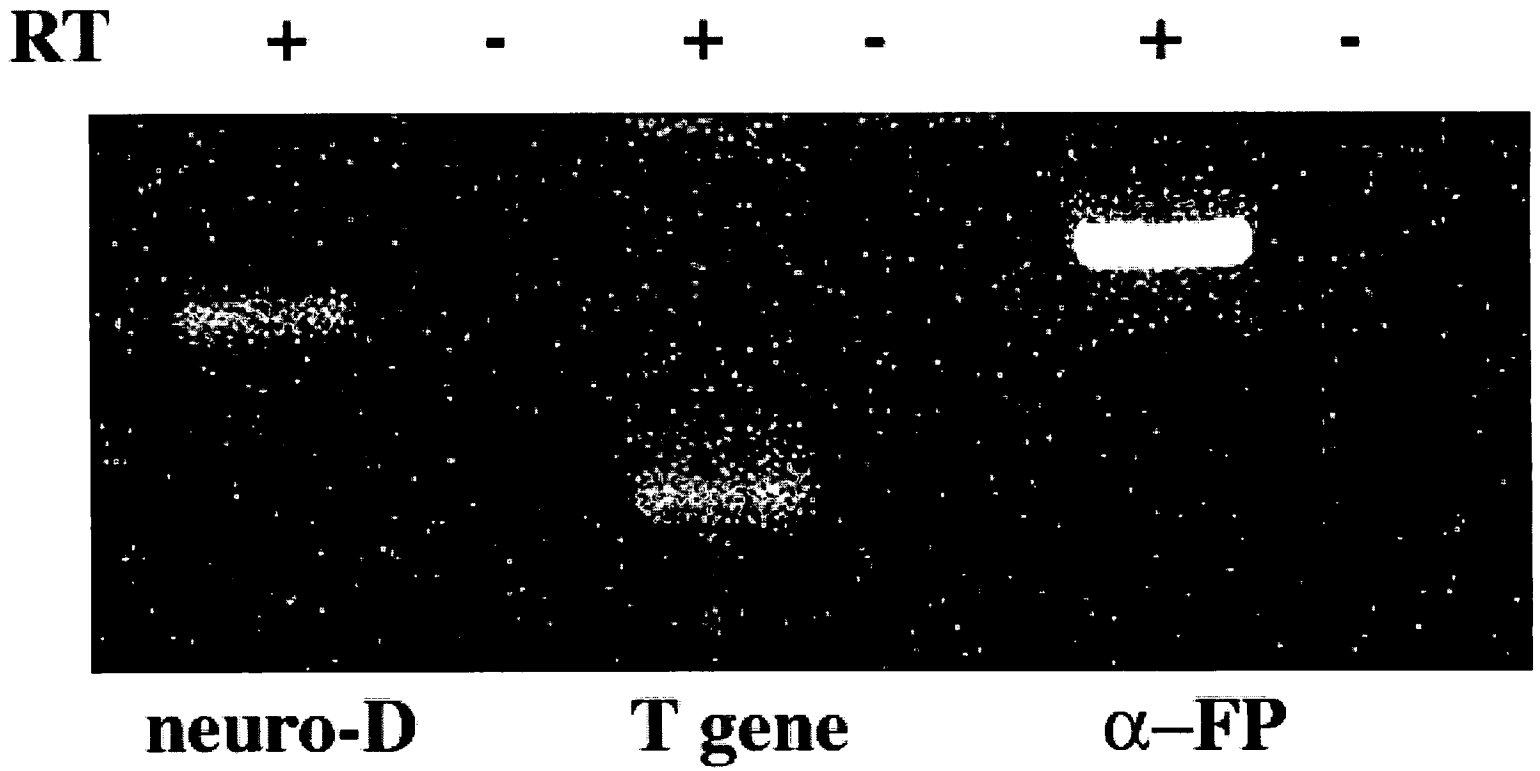

Fig. 2b

SEQ ID NO:01:
Inhibin beta A subunit (Activin A) Homo sapiens(PeproTech and GenBank X04447) macrophage cell line U937 (ATCC CRL 1539)) amino acid sequence:

GLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAGTSGSSLSFH
STVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS

Fig. 3

SEQ ID NO:02:
Inhibin beta A chain (Activin beta-A chain) Homo sapiens (GenBank X04447) 3'-region macrophage cell line U937 (ATCC CRL 1539)nucleic acid sequence:

ggcttggagtgtgatggcaaggtcaacatctgctgtaagaaacagttctttgtcagttt
caaggacatcggctggaatgactggatcattgctccctctggctatcatgccaactact
gcgagggtgagtgcccgagccatatagcaggcacgtccgggtcctcactgtccttccac
tcaacagtcatcaaccactaccgcatgcggggccatagccccttgccaacctcaaatc
gtgctgtgtgcccaccaagctgagacccatgtccatgttgtactatgatgatggtcaaa
acatcatcaaaaaggacattcagaacatgatcgtggaggagtgtgggtgctcatag

Fig. 4

SEQ ID NO:03:
Inhibin beta A chain (Activin beta-A chain) Homo sapiens (Swiss-Prot P08476) (GenBank M13436) (Erythroid differentiation protein) (EDF) ovarian amino acid sequence:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHSAAPDCPSCALAALPKDVPNSQPEMVEAVK
KHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEMNELME
QTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRTKVTIRLFQ
QQKHPQGSLDTGEEAEEVGLKGERSELLLSEKVVDARKSTWHVFPVSSSIQRLLDQGKS
SLDVRIACEQCQESGASLVLLGKKKKKEEEGEGKKKGGGEGGAGADEEKEQSHRPFLML
QARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGEC
PSHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKK
DIQNMIVEECGCS

SEQ ID NO:04: Inhibin B subunit - RECOMBINANT INHIBIN Patent: WO 8606076-A 14 23-OCT-1986 (GeneBank A14422) amino acid sequence:

ARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECP
SHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKD
IQNMIVEECGCS

SEQ ID NO:05: Inhibin B subunit in testis Homo sapiens (GeneBank X72498) amino acid sequence:

GLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAGTSGSSLSFH
STVINHYACGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS

SEQ ID NO:06: Inhibin B subunit erythroid differentiation protein mRNA (EDF), acute monocytic leukemia cell line THP-1, Homo sapiens (GeneBank J03634) amino acid sequence:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHSAAPDCPSCALAALPKDVPNSQPEMVEAVK
KHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEMNELME
QTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRTKVTIRLFQ
QQKHPQGSLDTGEEAEEVGLKGERSELLLSEKVVDARKSTWHVFPVSSSIQRLLDQGKS
SLDVRIACEQCQESGASLVLLGKKKKKEEEGEGKKKGGGEGGAGADEEKEQSHRPFLML
QARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGEC
PSHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKK
DIQNMIVEECGCS

Fig. 5

SEQ ID NO:07:
Inhibin beta A chain (Activin beta-A chain) (Swiss-Prot Swiss-Prot Q04998) (GenBank X69619; BC053527) - Mus musculus (Mouse) amino acid sequence:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHGSAPDCPSCALATLP
KDGPNSQPEMVEAVKKHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVE
IEDDIGRRAEMNELMEQTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLK
VPKANRTRTKVTIRLFQQQKHPQGSLDTGDEAEEMGLKGERSELLLSEKVVDARKSTW
HIFPVSSSIQRLLDQGKSSLDVRIACEQCQESGASLVLLGKKKKKEVDGDGKKKDGSD
GGLEEEKEQSHRPFLMLQARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWN
DWIIAPSGYHANYCEGECPSHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPT
KLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS

SEQ ID NO:08:
Inhibin beta A chain (Activin beta-A chain) (Swiss-Prot P18331) (GenBank M37482) - Rattus norvegicus (Rat) amino acid sequence:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHGAAPDCPSCALATLP
KDGPNSQPEMVEAVKKHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVE
IEDDIGRRAEMNELMEQTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLK
VPKANRTRTKVTIRLFQQQKHPQGSLDMGDEAEEMGLKGERSELLLSEKVVDARKSTW
HIFPVSSSIQRLLDQGKSSLDVRIACEQCQESGASLVLLGKKKKKEVDGDGKKKDGSD
GGLEEEKEQSHRPFLMLQARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWN
DWIIAPSGYHANYCEGECPSHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPT
KLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS

SEQ ID NO:09:
Inhibin beta A chain (Activin beta-A chain) (Swiss-Prot P27092)(GenBank U26946; U42377; M61167; M57407 - Gallus gallus (Chicken) amino acid sequence:

MPLLWKRGFLLVICWIIVRSSPTPGSEGHSSVADCPSCALTTLSKDVPSSQPEMVEAVK
KHILNMLHLRDRPNITQPVPKAALLNATKKLHVGKVGDDGYVEIEDDVGRRAEMNEVVE
QTSEIITFAESGTPKKTLHFEISKEGSELSVVEHAEVWLFLKVSKANRSRTKVTIRLFQ
QQRQPKGNSEAAEDMEDMGLKGERSETLISEKAVDARKSTWHIFPISSSVQRLLDQGQS
SLDVRIACDLCQETGASLVLLGKKKKKEDDGEGKEKDGGELTGEEEKEQSHRPFLMMLA
RHSEDRQHRRRERGLECDGKVNICCKKQFFVSFKDIGWSDWIIAPTGYHANYCEEECPS
HIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDI
QNMIVEECGCS

Fig. 6A

SEQ ID NO:10:
Inhibin beta A chain (Activin beta-A chain) (Swiss-Prot P07995) (GenBank U16239; U16238 JOINED; M13274) - Bos taurus (Bovine) amino acid sequence:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHSAAPDCPSCALATLPKDVPNSQPEMVEAVK
KHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEMNELME
QTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEIWLFLKVPKANRTRSKVTIRLFQ
QQKHLQGSLDAGEEAEEVGLKGEKSEMLISEKVVDARKSTWHIFPVSSCIQRLLDQGKS
SLDIRIACEQCQETGASLVLLGKKKKKEEEGEGKKRDGEGGAGGDEEKEQSHRPFLMLQ
ARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECP
SHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKD
IQNMIVEECGCS

SEQ ID NO:11:
Inhibin beta A chain (Activin beta-A chain) (Swiss-Prot P55102) (GenBank D50326) - Equus caballus (Horse) amino acid sequence:

MPLLWLRGFLLASCWIIVKSSPTPGSEGHSAAPNCPSCALATLPKDVPNAQPEMVEAVK
KHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEMNELME
QTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRSKVTIRLLQ
QQKHPQGSSDTREEAEEADLMEERSEQLISEKVVDARKSTWHIFPVSSSIQRLLDQGKS
SLDIRIACDQCHETGASLVLLGKKKKKEEEGEGKKKDGGEAGAGVDEEKEQSHRPFLML
QARQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGEC
PSHIAGTSGSSLSFHSTVINQYRLRGHNPFANLKSCCVPTKLRPMSMLYYDDGQNIIKK
DIQNMIVEECGCS

SEQ ID NO:12:
Inhibin beta A chain (Activin beta-A chain) (Swiss-Prot P03970) (GenBank X03266) - Sus scrofa (Pig) amino acid sequence:

MPLLWLRGFLLASCWIIVRSSPTPGSGGHSAAPDCPSCALATLPKDVPNSQPEMVEAVK
KHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVELEDDIGRRAEMNELME
QTSEIITFAEAGTARKTLRFEISKEGSDLSVVERAEIWLFLKVPKANRTRTKVSIRLFQ
QQRRPQGSADAGEEAEDVGFPEEKSEVLISEKVVDARKSTWHIFPVSSSIQRLLDQGKS
ALDIRTACEQCHETGASLVLLGKKKKKEEEAEGRKRDGEGAGVDEEKEQSHRPFLMLQA
RQSEEHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPS
HIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDI
QNMIVEECGCS

Fig. 6B

SEQ ID NO:13:
Inhibin beta A chain (Activin beta-A chain) (Swiss-Prot P43032) (GenBank L19218) - Ovis aries (Sheep) amino acid sequence:

MPLLWLRGFLLASCWIIVRSSPTPGSEGHSAAPDCPSCALATLPKDVPNSQPEMVEAVK
KHILNMLHLKKRPDVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEMNELME
QTSEIITFAESGTARKTLHFEISQEGSDLSVVERAEIWLFLKVPKANRTRSKVTIRLFQ
QQKHLQGSLDAGEEAEEVGLKGEKSEMLISEKVVDARKSTWHIFPVSSCIQRLLDQGKS
SLDIRIACEQCQETGASLVLLGKKKRKEEEGEGKKRDGEGGAGGDEEKEQSHRPFLMLQ
ARQSEDHPHRRRRRGLECDGKVNICCKKQFYVSFKDIGWNDWIIAPSGYHANYCEGECP
SHIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKD
IQNMIVEECGCS

SEQ ID NO:14:
Inhibin beta A chain (Activin beta-A chain (GenBank BC056742) - Felis catus (cat) amino acid sequence:
MPLLWLRGFLLASCWIIVRSSPTPGSEGPGAAPDCPSCALATLPKDVPNSQPEMVEAVK
KHILNMLHLKKRPEVTQPVPKAALLNAIRKLHVGKVGENGYVEIEDDIGRRAEMNELME
QTSEIITFAESGTARKTLHFEISKEGSDLSVVERAEVWLFLKVPKANRTRTKVTIQLLQ
KQPQGGVDAGEEAEEMGLMEERNEVLISEKVVDARKSTWHIFPVSSSIQRLLDQGKSSL
DVRIACEQCHETGASLVLLGKKKKKEEEGEGKKKDGGDGGAGADEDKEQSHRPFLMLQA
RQSEDHPHRRRRRGLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPS
HIAGTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDI
QNMIVEECGCS

SEQ ID NO:15:
Inhibin beta A chain (Activin beta-A chain (GenBank BC056742) - Danio rerio (zebrafish) amino acid sequence:

MSPLPLLSGILLLLIRSCSLSAMVTKGSLPMSEQQAGATVCPSCALARFRKGVSESEDE
GAQQDVVEAVKRHILNMLHLQERPNITHPVPRAALLNAIRKVHVGRVAKDGSVLIEDEA
SNRAETEQAEQTEIITFAETGEAPGIVNFLISKEGGEMSVVDQANVWIFLRLPKGNRTR
ANVNIRLLLQQGAGEKILAEKSVDTRRSGWHTFPASESVQSLLQRGGSTLSLRVSCPLC
ADARATPVLVSPGGSEREQSHRPFLMAVVRQMDELSLRRRRKRGLECDGKARVCCKRQF
YVNFKDIGWNDWIIAPSGYHANYCEGDCASNVASITGNSLSFHSTVISHYRIRGYSPFT
NIKSCCVPTRLRAMSMLYYNEEQKIVKKDIQNMIVEECGCS

Fig. 6C

SEQ ID NO:16:
Inhibin beta A chain (Activin beta-A chain (GenBank BC056742) - Carassius auratus (goldfish) amino acid sequence:

MSSLTLVNRGTAALRLFVRGLLTHSSREWLSGDGEPDDPVTPCP
SCALAQRQKDSEEQTDMVEAVKRHILNMLHLNTRPNVTHPVPRAALLNAIRRLHVGRV
GEDGTVEMEEDGGGLGEHREQSEEQPFEIITFAEPGDAPDIMKFDISMEGNTLSVVEQ
ANVWLLLKVAKGSRGKGKVSVQLLQHGKADPGSADGPQEAVVSEKTVDTRRSGWHTLP
VSRTVQTLLDGDSSMLSLRVSCPMCAEAGAVPILVPTESNKGKEREQSHRPFLMVVLK
PAEEHPHRRSKRGLECDGKIRVCCKRQFYVNFKDIGWSDWIIAPSGYHANYCEGDCPS
HVASITGSALSFHSTVINHYRMRGYSPFNNIKSCCVPTRLRAMSMLYYNEEQKIIKKD
IQNMIVEECGCS

Fig. 6D

SEQ ID NO:17:
Keratinocyte growth factor (PeproTech) - Homo sapiens (Human) amino acid sequence amino acid sequence:

MCNDMTPEQMATNVNCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYLRIDKRGKVKGTQ
EMKNNYNIMEIRTVAVGIVAIKGVESEFYLAMNKEGKLYAKKECNEDCNFKELILENHY
NTYASAKWTHNGGEMFVALNQKGIPVRGKKTKKEQKTAHFLPMAIT

SEQ ID NO:18:
Keratinocyte growth factor (Swiss-Prot P21781) (GenBank M60828; S81661) - Homo sapiens (Human) amino acid sequence:

MHKWILTWILPTLLYRSCFHIICLVGTISLACNDMTPEQMATNVNCSSPERHTRSYDYM
EGGDIRVRRLFCRTQWYLRIDKRGKVKGTQEMKNNYNIMEIRTVAVGIVAIKGVESEFY
LAMNKEGKLYAKKECNEDCNFKELILENHYNTYASAKWTHNGGEMFVALNQKGIPVRGK
KTKKEQKTAHFLPMAIT

SEQ ID NO:19:
Keratinocyte growth factor (Swiss-Prot P36363) (GenBank Z22703; U58503; BC052847) - Mus musculus (Mouse) amino acid sequence:

MRKWILTRILPTLLYRSCFHLVCLVGTISLACNDMSPEQTATSVNCSSPERHTRSYDYM
EGGDIRVRRLFCRTQWYLRIDKRGKVKGTQEMKNSYNIMEIRTVAVGIVAIKGVESEYY
LAMNKEGKLYAKKECNEDCNFKELILENHYNTYASAKWTHSGGEMFVALNQKGIPVKGK
KTKKEQKTAHFLPMAIT

SEQ ID NO:20:
Keratinocyte growth factor (Swiss-Prot P79150) (GenBank U80800) - Canis familiaris (Dog) amino acid sequence:

MRKWILTWILPTLLYRSCFHIICLVGTISLACNDMTPEQMATNV
NCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYLRIDKRGKVKGTQEMKNSYNIMEIRT
VAVGIVAIKGVESEYYLAMNKEGKLYAKKECNEDCNFKELILENHYNTYASAKWTHSG
GEMFVALNQKGVPVRGKKTKKEQKTAHFLPMAIT

Fig. 7A

SEQ ID NO:21:
Keratinocyte growth factor (Swiss-Prot Q9N198) (GenBank AF217463) - Sus scrofa (Pig) amino acid sequence:

MRKWILTWILPSLLHRSCFHIICLVGTLSLDCNDMTPEQMATNV
NCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYPRIGKRGKVKGTQEMKNNYNIMEIRT
VAVGIVAIKGVVSEYYLAMNKEGKLYAKKEYNEDCNFKELILENHYNTYASAKWTHSG
GEMFVALNQKGVPVRGKKTKKEQKTAHFLPMAIT

SEQ ID NO:22:
Keratinocyte growth factor (HBGF-7)(Swiss-Prot Q02195) (GenBank X56551) - Rattus norvegicus (Rat) amino acid sequence:

MRKWILTRILPTPLYRPCFHLVCLVGTISLACNDMSPEQTATSV
NCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYLRIDKRGKVKGTQEMRNSYNIMEIMT
VAVGIVAIKGVESEYYLAMNKQGELYAKKECNEDCNFKELILENHYNTSASAKWTHSG
GEMFVALNQKGLPVKGKKTKKEQKTAHFLPMAIT

SEQ ID NO:23:
Keratinocyte growth factor (Swiss-Prot P48808) (GenBank Z46236) - Ovis aries (Sheep) amino acid sequence:

MRKWILTWILPTLLYRSCFHIICLVGTISLACNDMTPEQMATNV
NCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYLRIDKRGKVKGTQEMKNSYNIMEIRT
VAVGIVAIKGVESEYYLAMNKEGKLYAKKECNEDCNFKELILENHYNTYASAKWTHSG
GEMFVALNQKGVPVRGKKTKKEQKTAHFLPMAIT

SEQ ID NO:24:
Keratinocyte growth factor (FGF-7) (GenBank AF420232) - Mustela vison (American mink) amino acid sequence
MRKWILTWILPTLLYRSCFHIICLVGTISLACNDMTPEQMATNV
NCSSPERHTRSYDYMEGGDIRVRRLFCRTQWYLRIDKRGKVKGTQEMKNSYNIMEIRT
VAVGIVAIKGVESEYYLAMNKEGKLYAKKECNEDCNFKELILENHYNTYASAKWTHSG
GEMFVALNQKGVPVRGKKTKKEQKQP

Fig. 7B

SEQ ID NO:25:
Keratinocyte growth factor (Swiss-Prot P21781) (GenBank S81661) - Homo sapiens (Human) nucleic acid sequence:

acgcgctcacacacagagagaaaatccttctgcctgttgatttatggaaacaattatga
ttctgctggagaacttttcagctgagaaatagtttgtagctacagtagaaaggctcaag
ttgcaccaggcagacaacagacatggaattcttatatatccagctgttagcaacaaaac
aaaagtcaaatagcaaacagcgtcacagcaactgaacttactacgaactgtttttatga
ggatttatcaacagagttatttaaggaggaatcctgtgttgttatcaggaactaaaagg
ataaggctaacaatttggaaagagcaagtactctttcttaaatcaatctacaattcaca
gataggaagaggtcaatgacctaggagtaacaatcaactcaagattcattttcattatg
ttattcatgaacacccggagcactacactataatgcacaaatggatactgacatggatc
ctgccaactttgctctacagatcatgctttcacattatctgtctagtgggtactatatc
tttagcttgcaatgacatgactccagagcaaatggctacaaatgtgaactgttccagcc
ctgagcgacacacaagaagttatgattacatggaaggaggggatataagagtgagaaga
ctcttctgtcgaacacagtggtacctgaggatcgataaaagaggcaaagtaaaagggac
ccaagagatgaagaataattacaatatcatggaaatcaggacagtggcagttggaattg
tggcaatcaaaggggtggaaagtgaattctatcttgcaatgaacaaggaaggaaaactc
tatgcaaagaaagaatgcaatgaagattgtaacttcaaagaactaattctggaaaacca
ttacaacacatatgcatcagctaaatggacacacaacggaggggaaatgtttgttgcct
taaatcaaaaggggattcctgtaagaggaaaaaaaacgaagaaagaacaaaaaacagcc
cactttcttcctatggcaataacttaattgcatatggtatataaagaacccagttccag
cagggagatttctttaagtggactgttttctttcttctcaaaattttctttccttttat
tttttagtaatcaagaaaggctggaaaaactactgaaaaactgatcaagctggacttgt
gcatttatgtttgttttaag

SEQ ID NO:26:
Keratinocyte growth factor (Swiss-Prot P36363) (GenBank Z22703) - Mus musculus (Mouse) nucleic acid sequence:

atgcgcaaatggatactgacacggatcctgccaactctgctctacagatcatgcttcca
cctcgtctgtctagtgggcactatatctctagcttgcaatgacatgagtccggagcaaa
cggctacgagtgtgaactgttccagccccgagcgacacaccagaagttatgactacatg
gaaggaggggatataagggtgagaagactgttctgtcgcacccagtggtacctgaggat
tgacaaacgaggcaaagtgaaagggacccaggagatgaagaacagctacaacatcatgg
aaatcaggaccgtggcagttggaattgtggcaatcaaaggggtggaaagtgaatactat
cttgccatgaacaaggaagggaaactctatgcaaagaaagaatgcaatgaggattgcaa
cttcaaagaactgattctggaaaaccattataacacctatgcatcagctaaatggacac
acagcggaggggaaatgttcgttgccttaaatcaaaaggggattcctgtcaaagggaag
aaaacgaagaaagaacaaaaaacagcccattttcttcctatggcaataacctaa

Fig. 8A

SEQ ID NO:27:
Keratinocyte growth factor (Swiss-Prot P79150) (GenBank U80800) - Canis familiaris (Dog) nucleic acid sequence:

agaggtcaatgacccaggagcaacaatcaactcaagatttaattttcattatgttattc
atgaacacccggagcactacactataatgcgcaaatggatactgacatggatcctgcca
actttgctctacagatcatgctttcacattatctgtctagtgggcactatatctttagc
ttgcaatgacatgactccagagcaaatggctacaaatgtgaactgttccagccctgagc
gacatacaagaagttatgattacatggaaggaggggatataagagtgagaagactcttc
tgtcgaacacagtggtatctgaggattgataaacgaggcaaagtcaaagggacccaaga
gatgaagaacagttacaatatcatggaaatcaggacagtggcagttggaatagtggcaa
tcaaaggggtggaaagtgaatattatcttgcaatgaataaggaaggaaagctctatgca
aagaaagaatgcaatgaagattgcaacttcaaagaattaattctggaaaaccattacaa
cacatatgcatcagctaaatggacacacagcggaggagaaatgtttgttgctttaaatc
aaaaggggttcctgtaaggggaaaaaaacgaagaaagaacaaaaaacagcccacttt
cttcctatggcaataacataatcatatatggtatata

SEQ ID NO:28:
Keratinocyte growth factor (Swiss-Prot Q9N198) (GenBank AF217463) - Sus scrofa (Pig) nucleic acid sequence:

aatctacaattcacagataggaagaggtcagtgacctaggagcaacgatcaactcaaga
tttattttcattatgttattcatgaacacccggagcactatactataatgcgcaaatgg
atactgacatggatcctgccaagtttgctccacagatcatgcttccacattatctgtct
ggtgggcactttatctttggattgcaatgacatgactccagagcaaatggctacaaatg
tgaactgttccagccctgagcgacatacaagaagttatgattacatggaaggaggggat
ataagagtgagaagactcttctgtcgaacacagtggtatccgaggattggcaaacgagg
caaagtcaaagggactcaagagatgaagaacaattacaacatcatggaaatcaggacag
tggctgttggaattgtagcaatcaaaggagtggtaagtgaatattatcttgcaatgaac
aaggaaggaaaactctatgcaaagaaagaatacaatgaagattgtaacttcaaagaatt
aattctggaaaaccattacaacacgtatgcatcagctaaatggacacacagtggaggag
aaatgtttgttgccttaaatcaaaaggggttcctgtaagagggaaaaaaaccaagaaa
gaacaaaaaacagcccactttcttcctatggcaataactaa

Fig. 8B

SEQ ID NO:29:
Keratinocyte growth factor (Swiss-Prot Q02195) (GenBank X56551) - Rattus norvegicus (Rat) nucleic acid sequence:

caatctacaattcacagataggaggaggcccatgacctaggagtagcgatcaactcaag
gtccagttctcattatgttattcatggacacccggggcactgctctataatgcgcaaat
ggatactgacacggatcctgccgactccgctctacagaccgtgcttccacctcgtctgt
cttgtgggcaccatatctttagcttgcaatgacatgagtccagagcagacggccacgag
cgtgaactgttctagccccgagcgacacacgagaagttatgactacatggaaggagggg
atataagggtgaggagactgttctgtcgcacccagtggtacctgaggattgacaaacga
ggcaaagtgaaagggacccaggagatgaggaacagctacaacatcatggaaatcatgac
tgtggcagttggaattgtggcaatcaaaggggtggaaagtgaatactatcttgccatga
acaaacaaggggaactctatgcaaagaaagaatgcaatgaggattgcaacttcaaagaa
ctgattctggaaaaccattacaacacctctgcatcagctaaatggacacacagcggagg
ggaaatgttcgtggccttaaatcaaaaggggcttcctgtcaaagggaagaaaacgaaga
aagaacaaaaaacggcccactttcttcctatggcaataacttaa

Fig. 8C

SEQ ID NO:30:
Keratinocyte growth factor (Swiss-Prot P48808) (GenBank Z46236) - Ovis aries (Sheep) nucleic acid sequence:

ttatgttattcatgaacacccggagcactatactataatgcgcaaatggatactgacat
ggatcctgccaagtttgctctacagatcatgcttccacattatctgtctagtgggcact
atatctttagcttgcaatgacatgactccagagcaaatggctacaaatgtgaactgttc
cagccccgagcgacatacaagaagttatgattacatggaaggaggagatataagagtga
gaagactcttctgtcgaacacagtggtatctgaggattgataaacgaggcaaagtcaaa
gggactcaagagatgaagaataattacaacatcatggaaatcaggacagtggctgttgg
aattgtagcaatcaaaggagtggaaagtgaatattaccttgcaatgaacaaggaaggaa
aactctatgcaaagaaagaatgtaacgaagactgcaacttcaaagaattaattctggaa
aatcattacaacacatatgcatcagctaaatggacacacagtggaggagaaatgtttgt
tgccttaaattcaaaaggggttccagtaagagggaagaaaacgaagaaagaacaaaaaa
cagcccactttcttcctatggcaataacttaa

SEQ ID NO:31:
Keratinocyte growth factor (FGF-7) (GenBank AF420232) - Mustela vison (American mink) nucleic acid sequence

atgcgcaaatggatactgacatggatcctgccaactttgctctacagatcatgctttca
cattatctgtctagtgggcactatatctttagcttgcaatgacatgactccagagcaaa
tggctacaaatgtgaactgttccagccctgagcgacatacaagaagttatgattacatg
gaaggaggggatataagagtgagaagactcttctgtcgaacacagtggtatctgaggat
tgataaacgaggcaaggtcaaaggaacccaagagatgaagaacagttacaatatcatgg
aaatcaggacagtggcagttggaattgtggcaatcaaaggggtggaaagtgaatattat
cttgcaatgaataaggaaggaaaactctatgcaaagaaagaatgcaatgaagattgcaa
cttcaaagaattaattctggaaaaccattacaacacatatgcatcagctaaatggacac
acagcggaggagaaatgtttgttgctttaaatcaaaaggggttcctgtaaggggaaa
aaaacgaagaaagaacaaaaacagccc

Fig. 8D

SEQ ID NO:32:
Inhibin beta A chain (Activin beta-A chain) Homo sapiens (GenBank M13436) (Erythroid differentiation protein) (EDF) ovarian amino acid sequence:

tgctccctgacagccacaaacctacagcactgactgcattcagagaggaacctgcaaac
aaaacttcacagaaaacttttgttcttgttccagagaatttgctgaagaggagaagga
aaaaaaaaacaccaaaaaaaaaaataaaaaaatccacacacacaaaaaacctgcgcgtg
agggggaggaaaagcagggccttttaaaaaggcaatcacaacaacttttgctgccagg
atgcccttgctttggctgagaggatttctgttggcaagttgctggattatagtgaggag
ttcccccaccccaggatccgaggggcacagcgcggcccccgactgtccgtcctgtgcgc
tggccgccctcccaaaggatgtacccaactctcagccagagatggtggaggccgtcaag
aagcacattttaaacatgctgcacttgaagaagagacccgatgtcacccagccggtacc
caaggcggcgcttctgaacgcgatcagaaagcttcatgtgggcaaagtcggggagaacg
ggtatgtggagatagaggatgacattggaaggagggcagaaatgaatgaacttatggag
cagacctcggagatcatcacgtttgccgagtcaggaacagccaggaagacgctgcactt
cgagatttccaaggaaggcagtgacctgtcagtggtggagcgtgcagaagtctggctct
tcctaaaagtccccaaggccaacaggaccaggaccaaagtcaccatccgcctcttccag
cagcagaagcacccgcagggcagcttggacacaggggaagaggccgaggaagtgggctt
aaaggggagaggagtgaactgttgctctctgaaaaagtagtagacgctcggaagagca
cctggcatgtcttccctgtctccagcagcatccagcggttgctggaccagggcaagagc
tccctggacgttcggattgcctgtgagcagtgccaggagagtggcgccagcttggttct
cctgggcaagaagaagaagaaagaagaggaggggaagggaaaaagaagggcggaggtg
aaggtggggcaggagcagatgaggaaaaggagcagtcgcacagacctttcctcatgctg
caggcccggcagtctgaagaccaccctcatcgccggcgtcggcggggcttggagtgtga
tggcaaggtcaacatctgctgtaagaaacagttctttgtcagtttcaaggacatcggct
ggaatgactggatcattgctccctctggctatcatgccaactactgcgagggtgagtgc
ccgagccatatagcaggcacgtccgggtcctcactgtccttccactcaacagtcatcaa
ccactaccgcatgcggggccatagccccttgccaacctcaaatcgtgctgtgtgccca
ccaagctgagacccatgtccatgttgtactatgatgatggtcaaaacatcatcaaaaag
gacattcagaacatgatcgtggaggagtgtgggtgctcatagagttgcccagcccaggg
ggaaagggagcaagagttgtccagagaagacagtggcaaaatgaagaaatttttaaggt
ttctgagttaaccagaaaaatagaaattaaaaacaaaaca

SEQ ID NO:33:
Inhibin B subunit - RECOMBINANT INHIBIN
Patent: WO 8606076-A 14 23-OCT-1986 (GenBank A14422):
gcccggcagtctgaagaccaccctcatcgccggcgtcggcggggcttggagtgtgatgg
caaggtcaacatctgctgtaagaaacagttctttgtcagtttcaaggacatcggctgga
atgactggatcattgctccctctggctatcatgccaactactgcgagggtgagtgcccg
agccatatagcaggcacgtccgggtcctcactgtccttccactcaacagtcatcaacca
ctaccgcatgcggggccatagccccttgccaacctcaaatcgtgctgtgtgcccacca
agctgagacccatgtccatgttgtactatgatgatggtcaaaacatcatcaaaaaggac
attcagaacatgatcgtggaggagtgtgggtgctcatagagttgcccagcccagggggga
aagggagcaaga

Fig. 9A

SEQ ID NO:34:
Nucleotide sequence coding for the mature subunit beta(A) inhibin in testis Homo sapiens (GenBank X72498):

ggcctggagtgcgacggcaaggtcaacatctgctgtaagaaacagttctttgtcagttt
caaggacatcggctggaatgactggatcattgctccctctggctatcatgccaactact
gcgagggtgagtgcccgagccatatagcaggcacgtccgggtcctcactgtccttccac
tcaacagtcatcaaccactacgcatgcggccatagccccttgccaacctcaaatcgtg
ctgtgtgcccaccaagctgagacccatgtccatgttgtactatgatgatggtcaaaaca
tcatcaaaaaggacattcagaacatgatcgtggaggagtgcgggtgctcctaa

SEQ ID NO:35:
Human erythroid differentiation protein mRNA (EDF), Original source text: acute monocytic leukemia cell line THP-1, Homo sapiens (GenBank J03634):

tccacacacacaaaaaacctgcgcgtgaggggggaggaaaagcagggcctttaaaaagg
caatcacaacaacttttgctgccaggatgcccttgctttggctgagaggatttctgttg
gcaagttgctggattatagtgaggagttcccccaccccaggatccgaggggcacagcgc
ggcccccgactgtccgtcctgtgcgctggccgccctcccaaaggatgtacccaactctc
agccagagatggtggaggccgtcaagaagcacattttaaacatgctgcacttgaagaag
agacccgatgtcacccagccggtacccaaggcggcgcttctgaacgcgatcagaaagct
tcatgtgggcaaagtcggggagaacgggtatgtggagatagaggatgacattggaagga
gggcagaaatgaatgaacttatggagcagacctcggagatcatcacgtttgccgagtca
ggaacagccaggaagacgctgcacttcgagatttccaaggaaggcagtgacctgtcagt
ggtggagcgtgcagaagtctggctcttcctaaaagtccccaaggccaacaggaccagga
ccaaagtcaccatccgcctcttccagcagcagaagcacccgcagggcagcttggacaca
ggggaagaggccgaggaagtgggcttaaaggggagaggagtgaactgttgctctctga
aaaagtagtagacgctcggaagagcacctggcatgtcttccctgtctccagcagcatcc
agcggttgctggaccagggcaagagctccctggacgttcggattgcctgtgagcagtgc
caggagagtggcgccagcttggttctcctgggcaagaagaagaagaaagaagaggaggg
ggaagggaaaaagaagggcggaggtgaaggtggggcaggagcagatgaggaaaaggagc
agtcgcacagacctttcctcatgctgcaggcccggcagtctgaagaccaccctcatcgc
cggcgtcggcggggcttggagtgtgatggcaaggtcaacatctgctgtaagaaacagtt
ctttgtcagtttcaaggacatcggctggaatgactggatcattgctccctctggctatc
atgccaactactgcgagggtgagtgcccgagccatatagcaggcacgtccgggtcctca
ctgtccttccactcaacagtcatcaaccactaccgcatgcggggccatagcccctttgc
caacctcaaatcgtgctgtgtgcccaccaagctgagacccatgtccatgttgtactatg
atgatggtcaaaacatcatcaaaaaggacattcagaacatgatcgtggaggagtgtggg
tgctcatagagttgcccagcccaggggaaagggagcaagagttgtccagagaagacag
tggcaaaatgaagaaatttttaaggtttctgagttaaccagaaaaatagaaattaaaaa
caaaacaaaacaaaaaaaaaaacaaaaaaaaacaaaagtaaattaaaaacaaacctgat
gaaacagatgaaacagatgaaggaagatgtggaaatcttagcctgccttagccagggct
cagagatgaagcagtgaagagacagattgggagggaaagggagaatggtgtacccttta
tttcttctgaaatcacactgatgacatcagttgtttaaacggggtattgtcctttcccc
ccttgaggttcccttgtgagcttgaatcaaccaatctgatctgcagtagtgtggactag
aacaacccaaatagcatctagaaagccatgagtttgaaagggcccatcacaggcacttt
cctagcctaat

Fig. 9B

DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **ACTIVIN A MAINTAINS PLURIPOTENTIALITY OF HUMAN EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER LAYERS**, the specification of which is attached hereto. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor: **Gillian Beattie**

Inventor's Signature: _____ Date: _____
Residence: 3545 Caminito El Rincon #237, San Diego 92130 Citizenship: United States of America
Post Office Address: 3545 Caminito El Rincon #237, San Diego 92130

Full Name of Second Joint Inventor: **Alberto Hayek**

Inventor's Signature: _____ Date: _____
Residence: 8818 Nottingham Place, La Jolla, 92037 Citizenship: United States of America
Post Office Address: 8818 Nottingham Place, La Jolla, 92037

Full Name of Third Joint Inventor: **Ana Lopez**

Inventor's Signature: _____ Date: _____
Residence: 11115 Adriatic Place, San Diego 92126 Citizenship: United States of America
Post Office Address: 11115 Adriatic Place, San Diego 92126

PATENT
Attorney Docket No.: **UCSD-08812**

ASSIGNMENT

WHEREAS, WE, Gillian Beattie, Alberto Hayek and Ana Lopez, hereinafter referred to as "ASSIGNOR", have invented certain new and useful improvements as described and set forth in the below-identified application for United States Letters Patent:

Title of Invention: **ACTIVIN A MAINTAINS PLURIPOTENTIALITY OF HUMAN EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER LAYERS**

Filing Date: Serial No.:

WHEREAS, The Regents of the University of California, a non-profit organization, 1111 Franklin Street, Oakland, California 94607, hereinafter referred to as "ASSIGNEE", is desirous of acquiring the entire right, title and interest in said invention and application and in any Letters Patent which may be granted on the same;

NOW THEREFORE, TO ALL WHOM IT MAY CONCERN: Be it known that, for and in consideration of the sum of One Dollar ($1.00) lawful money paid to ASSIGNOR by ASSIGNEE and other good and valuable consideration, receipt of which is hereby acknowledged, ASSIGNOR has sold, assigned and transferred, and by these presents does sell, assign and transfer unto said ASSIGNEE, and ASSIGNEE's successors and assigns, all right, title and interest in and to said invention, said application for United States Letters Patent, and any Letters Patent which may hereafter be granted on the same in the United States and all countries throughout the world including any divisions, renewals, continuations in whole or in part, substitutions, conversions, reissues, prolongations or extensions thereof, said interest to be held and enjoyed by said ASSIGNEE as fully and exclusively as it would have been held and enjoyed by said ASSIGNOR had this assignment and transfer not been made, to the full end and term of any Letters Patent.

ASSIGNOR further agrees that ASSIGNOR will, without charge to said ASSIGNEE, but at ASSIGNEE's expense, cooperate with ASSIGNEE in the prosecution of said application and/or applications; execute, verify, acknowledge and deliver all such further papers, including applications for Letters Patent and for the reissue thereof, and instruments of assignment and transfer thereof; and will perform such other acts as ASSIGNEE lawfully may request, to obtain or maintain Letters Patent for said invention and improvement in any and all countries, and to vest title thereto in said ASSIGNEE, or ASSIGNEE's successors and assigns.

IN TESTIMONY WHEREOF, ASSIGNOR has hereunto signed ASSIGNOR's names to this assignment on the date indicated below.

_____ _____

Gillian Beattie Alberto Hayek

_____

Ana Lopez

STATE OF _____)
) ss.
COUNTY OF _____)

On this _____ day of _____, in the year of _____, before me, the undersigned notary public, personally appeared the above-named ASSIGNOR, known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument, and acknowledged that he/she executed the same.

_____

NOTARY PUBLIC

SEAL

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Gillian Beattie *et al.* Group No.:
Serial No.: Examiner:
Filed:
Entitled: **ACTIVIN A MAINTAINS PLURIPOTENTIALITY OF HUMAN EMBRYONIC STEM CELLS IN THE ABSENCE OF FEEDER LAYERS**

POWER OF ATTORNEY BY ASSIGNEE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

The Regents of the University of California, as Assignee of record of the entire interest of the above-identified patent application, hereby appoints the members of the firm of MEDLEN & CARROLL, LLP, a firm composed of:

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as its attorneys with full power of substitution to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith.

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I hereby certify that the Assignment document filed with the application or filed subsequent to the filing date of the application, has been reviewed and I hereby certify that, to the best of my knowledge and belief, title is with The Regents of the University of California.

Dated: _____ By: _____

Name: _____

Title: _____

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